

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization International Bureau



(43) International Publication Date 15 May 2003 (15.05.2003)

PCT

(10) International Publication Number WO 03/040373 A2

(51) International Patent Classification7: C12N 15/52, 15/53, 15/54, 15/60, C12P 13/08, C12N 1/21 // (C12P 13/08, C12R 1:15)

MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ,

(21) International Application Number: PCT/EP02/08464

(22) International Filing Date:

30 July 2002 (30.07.2002)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data: 60/309,878

6 August 2001 (06.08.2001) US

(71) Applicant (for all designated States except US): DE-GUSSA AG [DE/DE]; Bennigsenplatz 1, 40474 Düsseldorf (DE).

(72) Inventors; and

- (75) Inventors/Applicants (for US only): BATHE, Brigitte [DE/DE]; Twieten 1, 33154 Salzkotten (DE). KREUTZER, Caroline [DE/DE]; Poststrasse 16, 49326 Melle (DE). MÖCKEL, Bettina [DE/DE]; Benrodestrasse 35, 40597 Düsseldorf (DE). THIERBACH, Georg [DE/DE]; Gunststrasse 21, 33613 Bielefeld (DE).
- (74) Common Representative: DEGUSSA AG; Intellectual Property Management, Patents And Trademarks, Location Hanau, P.O. Box 13 45, 63403 Hanau (DE).
- (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,

VN, YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

- as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW, ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, F1, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)
- of inventorship (Rule 4.17(iv)) for US only

Published:

without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: CORYNEFORM BACTERIA WHICH PRODUCE CHEMICAL COMPOUNDS I

(57) Abstract: The invention relates to coryneform bacteria which have, in addition to at least one copy, present at the natural site (locus), of an open reading frame (ORF), gene or allele which codes for the synthesis of a protein or an RNA, in each case a second, optionally third or fourth copy of this open reading frame (ORF), gene or allele at in each case a second, optionally third or fourth site in a form integrated into the chromosome and processes for the preparation of chemical compounds by fermentation of these bacteria.

Coryneform Bacteria which Produce Chemical Compounds I

Prior Art

30

Chemical compounds, which means, in particular, L-amino acids, vitamins, nucleosides and nucleotides and D-amino acids, are used in human medicine, in the pharmaceuticals industry, in cosmetics, in the foodstuffs industry and in animal nutrition.

Numerous of these compounds are prepared by fermentation from strains of coryneform bacteria, in particular

10 Corynebacterium glutamicum. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as, for example, stirring and supply of oxygen, or the composition of the nutrient media, such as, for example, the sugar concentration during the fermentation, or the working up to the product form by, for example, ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites or are auxotrophic for metabolites of regulatory importance and which produce the particular compounds are obtained in this manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of Corynebacterium strains, by amplifying individual biosynthesis genes and investigating the effect on production.

A common method comprises amplification of certain biosynthesis genes in the particular microorganism by means of episomally replicating plasmids. This procedure has the

disadvantage that during the fermentation, which in industrial processes is in general associated with numerous generations, the plasmids are lost spontaneously (segregational instability).

Another method comprises duplicating certain biosynthesis genes by means of plasmids which do not replicate in the particular microorganism. In this method, the plasmid, including the cloned biosynthesis gene, is integrated into the chromosomal biosynthesis gene of the microorganism 10 (Reinscheid et al., Applied and Environmental Microbiology 60(1), 126-132 (1994); Jetten et al., Applied Microbiology and Biotechnology 43(1):76-82 (1995)). A disadvantage of this method is that the nucleotide sequences of the plasmid and of the antibiotic resistance gene necessary for the selection remain in the microorganism. This is a 15 disadvantage, for example, for the disposal and utilization of the biomass. Moreover, the expert expects such strains to be unstable as a result of disintegration by "Campbell type cross over" in a corresponding number of generations such as are usual in industrial fermentations. 20

Object of the Invention

The inventors had the object of providing new measures for improved fermentative preparation chemical compounds using coryneform bacteria.

25 Summary of the Invention

Coryneform bacteria which produce chemical compounds, characterised in that these have, in addition to at least one copy, present at the natural site (locus), of an open reading frame (ORF), gene or allele which codes for the synthesis of a protein or an RNA, a second, optionally third or fourth copy of the open reading frame (ORF), gene or allele in question at a second, optionally third or fourth site in a form integrated into the chromosome, no

nucleotide sequence which is capable of/enables episomal replication or transposition in microorganisms and no nucleotide sequence(s) which impart(s) resistance to antibiotics being present at the second, optionally third or fourth site, and the second, optionally third or fourth site not relating to open reading frames (ORF), genes or alleles which are essential for the growth of the bacteria and the production of the desired compound.

The invention also provides processes for the preparation of one or more chemical compounds, in which the following steps are carried out:

- a) fermentation of coryneform bacteria,
- which have, in addition to at least one copy, a1) present at the natural site (locus), of an open 15 reading frame (ORF), gene or allele which codes for the synthesis of a protein or an RNA, a second, optionally third or fourth copy of this open reading frame (ORF), gene or allele at a second, optionally third or fourth site in a form 20 integrated into the chromosome, no nucleotide sequence which is capable of/enables episomal replication or transposition in microorganisms and no nucleotide sequence(s) which impart(s) resistance to antibiotics being present at the second, optionally third or fourth site, and the 25 second, optionally third or fourth site not relating to open reading frames (ORF), genes or alleles which are essential for the growth of the bacteria and the production of the desired compound, and 30
 - in which the intracellular activity of the corresponding protein is increased, in particular the nucleotide sequence which codes for this protein is over-expressed,

4

- b) concentration of the chemical compound(s) in the fermentation broth and/or in the cells of the bacteria,
- c) isolation of the chemical compound(s), optionally
- d) with constituents from the fermentation broth and/or the biomass to the extent of > (greater than) 0 to 100 wt.%.

The invention also provides processes for the preparation of one or more chemical compounds, which comprise the following steps:

15

20

25

30

a) fermentation of coryneform bacteria, in particular of the genus Corynebacterium, which have, in addition to the copy of an open reading frame (ORF), gene or allele present at the natural site (locus), in each case a second, optionally third or fourth copy of the open reading frame (ORF), gene or allele in question at in each case a second, optionally third or fourth site in integrated form, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular second, optionally third or fourth site,

under conditions which allow expression of the said open reading frames (ORF), genes or alleles

- b) concentration of the chemical compound(s) in the fermentation broth and/or in the cells of the bacteria,
- c) isolation of the chemical compound(s), optionally

25

d) with constituents from the fermentation broth and/or the biomass to the extent of > (greater than) 0 to 100%.

Detailed Description of the Invention

Chemical compounds are to be understood, in particular, as meaning amino acids, vitamins, nucleosides and nucleotides. The biosynthesis pathways of these compounds are known and are available in the prior art.

Amino acids mean, preferably, L-amino acids, in particular
the proteinogenic L-amino acids, chosen from the group
consisting of L-aspartic acid, L-asparagine, L-threonine,
L-serine, L-glutamic acid, L-glutamine, glycine, L-alanine,
L-cysteine, L-valine, L-methionine, L-isoleucine, Lleucine, L-tyrosine, L-phenylalanine, L-histidine, Llysine, L-tryptophan, L-proline and L-arginine and salts
thereof, in particular L-lysine, L-methionine and Lthreonine. L-Lysine is very particularly preferred.

Proteinogenic amino acids are understood as meaning the amino acids which occur in natural proteins, that is to say in proteins of microorganisms, plants, animals and humans.

Vitamins mean, in particular, vitamin B1 (thiamine), vitamin B2 (riboflavin), vitamin B5 (pantothenic acid), vitamin B6 (pyridoxines), vitamin B12 (cyanocobalamin), nicotinic acid/nicotinamide, vitamin M (folic acid) and vitamin E (tocopherol) and salts thereof, pantothenic acid being preferred.

Nucleosides and nucleotides mean, inter alia, S-adenosyl-methionine, inosine-5'-monophosphoric acid and guanosine-5'-monophosphoric acid and salts thereof.

The coryneform bacteria are, in particular, those of the genus Corynebacterium. Of the genus Corynebacterium, the species Corynebacterium glutamicum, Corynebacterium

5

ammoniagenes and Corynebacterium thermoaminogenes are preferred. Information on the taxonomic classification of strains of this group of bacteria is to be found, inter alia, in Kämpfer and Kroppenstedt (Canadian Journal of Microbiology 42, 989-1005 (1996)) and in US-A-5,250,434.

Suitable strains of the species Corynebacterium glutamicum (C. glutamicum) are, in particular, the known wild-type strains

Corynebacterium glutamicum ATCC13032 10 Corynebacterium acetoglutamicum ATCC15806 Corynebacterium acetoacidophilum ATCC13870 Corynebacterium lilium ATCC15990 Corynebacterium melassecola ATCC17965 Corynebacterium herculis ATCC13868 15 Arthrobacter sp. ATCC243 Brevibacterium chang-fua ATCC14017 Brevibacterium flavum ATCC14067 Brevibacterium lactofermentum ATCC13869 Brevibacterium divaricatum ATCC14020 20 Brevibacterium taipei ATCC13744 and Microbacterium ammoniaphilum ATCC21645

and mutants or strains, such as are known from the prior art, produced therefrom which produce chemical compounds.

Suitable strains of the species Corynebacterium

ammoniagenes (C. ammoniagenes) are, in particular, the known wild-type strains

Brevibacterium ammoniagenes ATCC6871 Brevibacterium ammoniagenes ATCC15137 and Corynebacterium sp. ATCC21084

and mutants or strains, such as are known from the prior art, produced therefrom which produce chemical compounds.

Suitable strains of the species Corynebacterium thermoaminogenes (C. thermoaminogenes) are, in particular, the known wild-type strains

Corynebacterium thermoaminogenes FERM BP-1539
Corynebacterium thermoaminogenes FERM BP-1540
Corynebacterium thermoaminogenes FERM BP-1541 and
Corynebacterium thermoaminogenes FERM BP-1542

and mutants or strains, such as are known from the prior art, produced therefrom which produce chemical compounds.

5

Strains with the designation "ATCC" can be obtained from the American Type Culture Collection (Manassas, VA, USA). Strains with the designation "FERM" can be obtained from the National Institute of Advanced Industrial Science and Technology (AIST Tsukuba Central 6, 1-1-1 Higashi, Tsukuba Ibaraki, Japan). The strains of Corynebacterium thermoaminogenes mentioned (FERM BP-1539, FERM BP-1540, FERM BP-1541 and FERM BP-1542) are described in US-A 5,250,434.

Open reading frame (ORF) describes a section of a nucleotide sequence which codes or can code for a protein or polypeptide or ribonucleic acid to which no function can be assigned according to the prior art.

After assignment of a function to the nucleotide sequence section in question, it is in general referred to as a gene.

Alleles are in general understood as meaning alternative forms of a given gene. The forms are distinguished by differences in the nucleotide sequence.

In the context of the present invention, endogenous, that is to say species-characteristic, open reading frames, genes or alleles are preferably used. These are understood as meaning the open reading frames, genes or alleles or

nucleotide sequences thereof present in the population of a species, such as, for example, Corynebacterium glutamicum.

"A copy of an open reading frame (ORF), a gene or allele present at the natural site (locus)" in the context of this invention is understood as meaning the position or situation of the ORF or gene or allele in relation to the adjacent ORFs or genes or alleles such as exists in the corresponding wild-type or corresponding parent organism or starting organism.

Thus, for example, the natural site of the lysC gene or of an lysC^{FBR} allele, which codes for a "feed back" resistant aspartate kinase from Corynebacterium glutamicum is the lysC site or lysC locus or lysC gene site with the directly adjacent genes or open reading frames orfX and leuA on one flank and the asd gene on the other flank.

"Feed back" resistant aspartate kinase is understood as meaning aspartate kinases which, compared with the wild-type form, have a lower sensitivity to inhibition by mixtures of lysine and threonine or mixtures of AEC (aminoethylcysteine) and threonine or lysine by itself or AEC by itself. Strains which produce L-lysine typically contain such "feed back" resistant or desensitized aspartate kinases.

20

The nucleotide sequence of the chromosome of

Corynebacterium glutamicum is known and can be found in Patent Application EP-A-1108790 and Access Number (Accession No.) AX114121 of the nucleotide sequence databank of the European Molecular Biologies Laboratories (EMBL, Heidelberg, Germany and Cambridge, UK). The nucleotide sequences of orfX, the leuA gene and the asd gene have the Access Numbers AX120364 (orfX), AX123517 (leuA) and AX123519 (asd).

9

5

Further databanks, such as, for example, that of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) or that of the Swiss Institute of Bioinformatics (Swissprot, Geneva, Switzerland) or that of the Protein Information Resource Database (PIR, Washington, DC, USA) can also be used.

"In each case a second, optionally third or fourth site" is understood as meaning a site which differs from the "natural site". It is also called a "target site" or "target sequence" in the following. It can also be called an "integration site" or "transformation site". This second, optionally third or fourth site, or the nucleotide sequence present at the corresponding sites, is preferably in the chromosome and is in general not essential for growth and for production of the desired chemical compounds.

To produce the coryneform bacteria according to the invention, the nucleotide sequence of the desired ORF, gene or allele, optionally including expression and/or 20 regulation signals, is isolated and provided with nucleotide sequences of the target site at the ends, these are then transferred into the desired coryneform bacterium, preferably with the aid of vectors which do not replicate or replicate to only a limited extent in coryneform 25 bacteria, and those bacteria in which the desired ORF, gene or allele is incorporated at the target site are isolated, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining 30 at the target site.

The invention accordingly also provides a process for the production of coryneform bacteria which produce one or more chemical compounds, which comprises

- a) isolating the nucleotide sequence of at least one desired ORF, gene or allele, optionally including the expression and/or regulation signals,
- b) providing the 5' and the 3' end of the ORF, gene or allele with nucleotide sequences of the target site,

10

25

- c) preferably incorporating the nucleotide sequence of the desired ORF, gene or allele provided with nucleotide sequences of the target site into a vector which does not replicate or replicates to only a limited extent in coryneform bacteria,
- d) transferring the nucleotide sequence according to b) or c) into coryneform bacteria, and
- e) isolating coryneform bacteria in which the nucleotide sequence according to a) is incorporated at the target site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the target site.

Preferably, also, no residues of sequences of the vectors used or species-foreign DNA, such as, for example, restriction cleavage sites, remain at the target site. A maximum of 24, preferably a maximum of 12, particularly preferably a maximum of 6 nucleotides of such DNA upstream or downstream of the ORF, gene or allele incorporated optionally remain at the target site.

By the measures according to the invention, the productivity of the coryneform bacteria or of the fermentative processes for the preparation of chemical compounds is improved in respect of one or more of the features chosen from the group consisting of concentration (chemical compound formed, based on the unit volume), yield

(chemical compound formed, based on the source of carbon consumed) and product formation rate (chemical compound formed, based on the time) by at least 0.5 - 1.0% or at least 1.0 to 1.5% or at least 1.5 - 2.0%.

Instructions on conventional genetic engineering methods, such as, for example, isolation of chromosomal DNA, plasmid DNA, handling of restriction enzymes etc., are found in Sambrook et al. (Molecular Cloning - A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press). Instructions on transformation and conjugation in coryneform bacteria are found, inter alia, in Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), in Schäfer et al. (Journal of Bacteriology 172, 1663-1666 (1990) and Gene 145, 69-73 (1994)) and in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)).

Vectors which replicate to only a limited extent are understood as meaning plasmid vectors which, as a function of the conditions under which the host or carrier is cultured, replicate or do not replicate. Thus, a temperature-sensitive plasmid for coryneform bacteria which can replicate only at temperatures below 31°C has been described by Nakamura et al. (US-A-6,303,383).

20

30

The invention furthermore provides coryneform bacteria, in particular of the genus Corynebacterium, which produce L-lysine, characterized in that these have, in addition to at least one of the copy of an open reading frame (ORF), gene or allele of lysine production present at the natural site (locus), in each case a second, optionally third or fourth copy of the open reading frame (ORF), gene or allele in question at in each case a second, optionally third or fourth site in integrated form, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which

imparts resistance to antibiotics being present at the particular second, optionally third or fourth site.

The invention also furthermore provides a process for the preparation of L-lysine, which comprises the following steps:

5

25

30

- fermentation of coryneform bacteria, in particular a) Corynebacterium glutamicum, characterized in that these have, in addition to at least one of the copy of an open reading frame (ORF), gene or allele of lysine production present at the natural site 10 (locus), in each case a second, optionally third or fourth copy of the open reading frame (ORF), gene or allele in question at in each case a second, optionally third or fourth site in integrated form, no nucleotide sequence which is capable of/enables 15 episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular second, optionally third or fourth site, 20
 - under conditions which allow expression of the said open reading frames (ORF), genes or alleles,
 - b) concentration of the L-lysine in the fermentation broth,
 - c) isolation of the L-lysine from the fermentation broth, optionally
 - d) with constituents from the fermentation broth and/or the biomass to the extent of > (greater than) 0 to 100%.

A "copy of an open reading frame (ORF), gene or allele of lysine production" is to be understood as meaning all the, preferably endogenous, open reading frames, genes or

alleles of which enhancement/over-expression can have the effect of improving lysine production. Enhancement is understood as meaning an increase in the intracellular concentration or activity of the particular gene product, protein or enzyme.

These include, inter alia, the following open reading frames, genes or alleles: accBC, accDA, cstA, cysD, cysE, cysH, cysK, cysN, cysQ, dapA, dapB, dapC, dapD, dapE, dapF, ddh, dps, eno, gap, gap2, gdh, gnd, lysC, lysCFBR, lysE, msiK, opcA, oxyR, ppc, ppcFBR, pgk, pknA, pknB, pknD, pknG, ppsA, ptsH, ptsI, ptsM, pyc, pyc P458S, sigC, sigD, sigE, sigH, sigM, tal, thyA, tkt, tpi, zwa1, zwf and zwf A213T. These are summarized and explained in Table 1.

These include, in particular, the lysC^{FBR} alleles which code for a "feed back" resistant aspartate kinase. Various lysC^{FBR} alleles are summarized and explained in Table 2.

The following lysCFBR alleles are preferred: lysC A279T (replacement of alanine at position 279 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by threonine), lysC A279V (replacement of alanine at position 279 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by valine), lysC S301F (replacement of serine at position 301 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by phenylalanine), lysC T308I 25 (replacement of threonine at position 308 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by isoleucine), lysC S301Y (replacement of serine at position 308 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by tyrosine), lysC G345D (replacement of glycine at position 345 of the aspartate kinase protein coded, 30 according to SEQ ID NO: 2, by aspartic acid), lysC R320G (replacement of arginine at position 320 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by glycine), lysC T311I (replacement of threonine at position 35 311 of the aspartate kinase protein coded, according to SEQ

ID NO: 2, by isoleucine), lysC S381F (replacement of serine at position 381 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by phenylalanine).

The lysC^{FBR} allele lysC T311I (replacement of threonine at position 311 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by isoleucine), the nucleotide sequence of which is shown as SEQ ID NO:3, is particularly preferred; the amino acid sequence of the aspartate kinase protein coded is shown as SEQ ID NO:4.

- The second, optionally third or fourth copy of the open reading frame (ORF), gene or allele of lysine production in question can be integrated at in each case a second, optionally third or fourth site. The following open reading frames, genes or nucleotide sequences, inter alia, can be used for this: aecD, ccpA1, ccpA2, citA, citB, citE, fda, gluA, gluB, gluC, gluD, luxR, luxS, lysR1, lysR2, lysR3, menE, mqo, pck, pgi, poxB and zwa2, in particular the genes aecD, gluA, gluB, gluC, gluD and pck. These are summarized and explained in Table 3.
- The sites mentioned include, of course, not only the coding regions of the open reading frames or genes mentioned, but also the regions or nucleotide sequences lying upstream which are responsible for expression and regulation, such as, for example, ribosome binding sites, promoters, binding sites for regulatory ribonucleic acids and attenuators. These regions in general lie in a range of 1-800, 1-600, 1-400, 1-200, 1-100 or 1-50 nucleotides upstream of the coding region. In the same way, regions lying downstream, such as, for example,
- transcription terminators, are also included. These regions in general lie in a range of 1-400, 1-200, 1-100, 1-50 or 1-25 nucleotides downstream of the coding region.

Intergenic regions in the chromosome, that is to say nucleotide sequences without a coding function, can

furthermore be used. Finally, prophages or defective phages contained in the chromosome can be used for this.

A prophage is understood as meaning a bacteriophage, in particular the genome thereof, where this is replicated together with the genome of the host and the formation of infectious particles does not take place. A defective phage is understood as meaning a prophage, in particular the genome thereof, which, as a result of various mutations, has lost the ability to form so-called infectious 10 particles. Defective phages are also called cryptic. Prophages and defective phages are often present in integrated form in the chromosome of their host. Further details exist in the prior art, for example in the textbook by Edward A. Birge (Bacterial and Bacteriophage Genetics, 3rd ed., Springer-Verlag, New York, USA, 1994) or in the 15 textbook by S. Klaus et al. (Bakterienviren, Gustav Fischer

Verlag, Jena, Germany, 1992).

Table 1
Open reading frames, genes and alleles of lysine production

Name	Description of the coded enzyme or	Reference	Access
	protein		Number
}			
accBC	Acyl-CoA Carboxylase	Jäger et al.	บ35023
	EC 6.3.4.14	Archives of	
	(acyl-CoA carboxylase)	Microbiology	
	(acyr-con carboxyrase)	(1996) 166:76-	
		82	
		EP1108790;	AX123524
		WO0100805	AX066441
accDA	Acetyl-CoA Carboxylase	EP1055725	727000172
accon	EC 6.4.1.2	EP1108790	AX121013
	(acetyl-CoA carboxylase)	WO0100805	AX066443
cstA	Carbon Starvation Protein A	EP1108790	AX120811
CSLA		WO0100804	AX066109
	(carbon starvation protein A)	EP1108790	AX123177
cysD	Sulfate Adenylyltransferase	EF1100/90	RAIZJIII
	sub-unit II	4	
	EC 2.7.7.4		
	(sulfate adenylyltransferase small		
	chain)	EP1108790	AX122902
cysE	Serine Acetyltransferase	WO0100843	AX063961
	EC 2.3.1.30	MO01009#2	W002301
	(serine acetyltransferase)	EP1108790	AX123178
cysH .	3'-Phosphoadenyl Sulfate Reductase	WO0100842	AX066001
	EC 1.8.99.4	WO0100842	ANUGUUI
	(3'-phosphoadenosine 5'-		
	phosphosulfate reductase)	EP1108790	AX122901
cysK	Cysteine Synthase	WO0100843	AX063963
	EC 4.2.99.8	MOOTOOGED	ANOUSSUS
NT	(cysteine synthase)	EP1108790	AX123176
cysN	Sulfate Adenylyltransferase sub- unit I	DE 1700120	AX127152
	EC 2.7.7.4		AMILITA
	(sulfate adenylyltransferase)		
		EP1108790	AX127145
cysQ	Transport Protein CysQ	WO0100805	AX066423
2003	(transporter cysQ)	Bonnassie et	X53993
dapA	Dihydrodipicolinate Synthase EC 4.2.1.52	al. Nucleic	AJJJJ
	(dihydrodipicolinate synthase)	Acids Research	
	(dinydrodipicolinace synthase)	18:6421 (1990)	
		Pisabarro et	
		al., Journal of	
		Bacteriology	Z21502
		175:2743-	221702
		2749 (1993)	
		EP1108790	}
		WO0100805	[
		EP0435132	<u> </u>
		EP0435132 EP1067192	AX123560
		EP1067192 EP1067193	AX063773
Acro D	Dibududini adlimate Deducter	EP1007193	AX127149
dapB	Dihydrodipicolinate Reductase EC 1.3.1.26	WO0100843	AX127149 AX063753
		EP1067192	AX137723
	(dihydrodipicolinate reductase)	ELIVOITA	[EVT71152]

		EP1067193	AX137602
	·	Pisabarro et	x67737
		al., Journal of	Z21502
		Bacteriology	
		175:2743-	
1		2749 (1993)	1
		JP1998215883	E16749
		JP1997322774	E14520
		JP1997070291	E12773
		JP1995075578	E08900
dapC	N-Succinyl Aminoketopimelate	EP1108790	AX127146
	Transaminase	WO0100843	AX064219
	EC 2.6.1.17	EP1136559	AX004213
l:	(N-succinyl diaminopimelate	EE1120223	
	transaminase)		
dapD	Tetrahydrodipicolinate Succinylase	EP1108790	AX127146
	EC 2.3.1.117	WO0100843	AX063757
	(tetrahydrodipicolinate	Wehrmann et al.	AJ004934
	succinylase)	Journal of	
		Bacteriology	
		180:3159-	
	•	3165 (1998)	
dapE	N-Succinyl Diaminopimelate	EP1108790	AX127146
	Desuccinylase	W00100843	AX063749
	EC 3.5.1.18	Wehrmann et al.	X81379
	(N-succinyl diaminopimelate	Microbiology	A01379
	desuccinylase)	140:3349-3356	
		(1994)	
dapF	Diaminopimelate Epimerase	EP1108790	AX127149
	EC 5.1.1.7	WO0100843	AX063719
	(diaminopimelate epimerase)	EP1085094	AX137620
ddh	Diaminopimelate Dehydrogenase	EP1108790	AX127152
	EC 1.4.1.16	WO0100843	AX127152 AX063759
	(diaminopimelate dehydrogenase)	Ishino et al.,	Y00151
	(dramitroprimerace deligatogenase)	Nucleic Acids	100121
		1	
		Research	
		15:3917-	
		3917 (1987)	
		JP1997322774	E14511
		JP1993284970	E05776
		Kim et al.,	D87976
		Journal of	
		Microbiology	
		and	
,		Biotechnology	
		5:250-256(1995)	
dps	DNA Protection Protein	EP1108790	AX127153
	(protection during starvation		
	protein)		
eno	Enolase	EP1108790	AX127146
	EC 4.2.1.11	WO0100844	AX064945
	(enolase)	EP1090998	AX136862
		Hermann et al.,	
i		Electrophoresis	
		19:3217-3221	
		(1998)	
gap	Glyceraldehyde 3-Phosphate	EP1108790	AX127148
-	Dehydrogenase	WO0100844	AX064941
		1	

ŗ -	TO 1 2 1 12	T	
	EC 1.2.1.12	Eikmanns et	X59403
	(glyceraldehyde 3-phosphate	al., Journal of	
	dehydrogenase)	Bacteriology	
		174:6076~	
		6086 (1992)	
gap2	Glyceraldehyde 3-Phosphate	EP1108790	AX127146
	Dehydrogenase	W00100844	AX064939
	EC 1.2.1.12	ļ	
	(glyceraldehyde 3-phosphate		
	dehydrogenase 2)		
gdh	Glutamate Dehydrogenase	EP1108790	AX127150
	EC 1.4.1.4	WO0100844	AX063811
	(glutamate dehydrogenase)	Boermann et	X59404
		al., Molecular	
		Microbiology	
		6:317-326	
		(1992).	
		Guyonvarch et	X72855
		al., NCBI	
gnd	6-Phosphogluconate Dehydrogenase	EP1108790	AX127147
	EC 1.1.1.44		AX121689
	(6-phosphogluconate dehydrogenase)	WO0100844	AX065125
lysC	Aspartate Kinase	EP1108790	AX120365
	EC 2.7.2.4	W00100844	AX063743
	(aspartate kinase)	Kalinowski et	X57226
		al., Molecular	
		Microbiology	
		5:1197-204	
- PDD		(1991)	
lysCFBR	Aspartate Kinase feedback resistant	see Table 2	
	(fbr)		
	EC 2.7.2.4		•
7	(aspartate kinase fbr)		
lysE	Lysine Exporter	EP1108790	AX123539
	(lysine exporter protein)	W00100843	AX123539
		Vrljić et al.,	X96471
		Molecular	
		Microbiology	
		22:815-826	
msiK	Cugar Importor	(1996)	
MSIK	Sugar Importer (multiple sugar import protein)	EP1108790	AX120892
opcA	Glucose 6-phosphate Dehydrogenase	W00104205	7**07.607.6
ODCA		WO0104325	AX076272
	(subunit of glucose 6-phosphate		
oxyR	dehydrogenase)	PD1100700	2
OXYK	Transcription Regulator	EP1108790	AX122198
ppcFBR	(transcriptional regulator)	FD0702011	AX127149
ppc	Phosphoenol Pyruvate Carboxylase	EP0723011	
	feedback resistant	WO0100852	
	EC 4.1.1.31		
	(phosphoenol pyruvate carboxylase		ŀ
nna	feedback resistant)		
ppc	Phosphoenol Pyruvate Carboxylase	EP1108790	AX127148
	EC 4.1.1.31		AX123554
ĺ	(phosphoenol pyruvate carboxylase)	O'Reagan et	M25819
	-	al., Gene	
			<u>-</u>
		77(2):237- 251(1989)	

pgk	Phosphoglycerate Kinase	EP1108790	AX121838
	EC 2.7.2.3	WO0100844	AX127148 AX064943
	(phosphoglycerate kinase)	Eikmanns,	X59403
		Journal of	LJ3403
		Bacteriology	
		174:6076-6086	
		(1992)	
pknA	Protein Kinase A	EP1108790	AX120131
	(protein kinase A)		AX120085
pknB	Protein Kinase B	EP1108790	AX120130
	(protein kinase B)		AX120085
pknD	Protein Kinase D	EP1108790	AX127150
	(protein kinase D)		AX122469
			AX122468
pknG	Protein Kinase G	EP1108790	AX127152
	(protein kinase G)		AX123109
ppsA	Phosphoenol Pyruvate Synthase	EP1108790	AX127144
	EC 2.7.9.2		AX120700
	(phosphoenol pyruvate synthase)		AX122469
ptsH	Phosphotransferase System Protein H	EP1108790	AX122210
	EC 2.7.1.69	7700100044	AX127149
į	(phosphotransferase system component H)	WO0100844	AX069154
ptsI	Phosphotransferase System Enzyme I	EP1108790	AX122206
DC31	EC 2.7.3.9	ППТТООТО	AX127149
	(phosphotransferase system		
	enzyme I)		
ptsM	Glukose-specific Phosphotransferase.	Lee et al.,	L18874
	System Enzyme II	FEMS	
	EC 2.7.1.69	Microbiology	
	(glucose phosphotransferase system	Letters 119	
	enzyme II)	(1-2):137-145	
		(1994)	20000
рус	Pyruvate Carboxylase	WO9918228	A97276
	EC 6.4.1.1	Peters-Wendisch et al.,	Y09548
	(pyruvate carboxylase)	Microbiology	
		144:915-927	ì
		(1998)	
рус	Pyruvate Carboxylase	EP1108790	
P458S	EC 6.4.1.1		
	(pyruvate carboxylase)	•	
	amino acid exchange P458S		
sigC	Sigma Factor C	EP1108790	AX120368
	EC 2.7.7.6		AX120085
	(extracytoplasmic function		
	alternative sigma factor C)		
sigD	RNA Polymerase Sigma Factor D	EP1108790	AX120753
	EC 2.7.7.6		AX127144
	(RNA polymerase sigma factor)	TD1100700	33105146
sigE	Sigma Factor E	EP1108790	AX127146
	EC 2.7.7.6		AX121325
	(extracytoplasmic function		
Ci att	alternative sigma factor E)	EP1108790	AX127145
sigH	Sigma Factor H EC 2.7.7.6	 551100130	AX127145 AX120939
	(sigma factor SigH)		
	Libration Locot Digit/		<u></u>

		44.00000	37122500
sigM	Sigma Factor M	EP1108790	AX123500
	EC 2.7.7.6		AX127145
	(sigma factor SigM)		
tal	Transaldolase	WO0104325	AX076272
	EC 2.2.1.2		
	(transaldolase)		
thyA	Thymidylate Synthase	EP1108790	AX121026
_	EC 2.1.1.45	,	AX127145
	(thymidylate synthase)		
tkt	Transketolase	Ikeda et al.,	AB023377
	EC 2.2.1.1	NCBI	
	(transketolase)		
tpi	Triose Phosphate Isomerase	Eikmanns,	X59403
_	EC 5.3.1.1	Journal of	
	(triose phosphate isomerase)	Bacteriology	
		174:6076-6086	
		(1992)	
zwa1	Cell Growth Factor 1	EP1111062	AX133781
	(growth factor 1)		
zwf	Glucose 6-phosphate 1-Dehydrogenase	EP1108790	AX127148
	EC 1.1.1.49		AX121827
E .	(glucose 6-phosphate 1-	WO0104325	AX076272
{	dehydrogenase)		
zwf	Glucose 6-phosphate 1-Dehydrogenase	EP1108790	
A213T	EC 1.1.1.49		
	(glucose 6-phosphate 1-		
	dehydrogenase)		
	amino acid exchange A213T		

lysC^{PBR} alleles which code for feed back resistant aspartate kinases

Name of the	Further	Reference	Access Number
allele	information	1,62,62,61.66	
lysC ^{PER} -E05108	Intornacion	JP 1993184366-A	E05108
1,00 205100		(sequence 1)	
lysCFBR-E06825	lysC A279T	JP 1994062866-A	E06825
1,50 20023	1,00	(sequence 1)	
lysCFBR-E06826	lysC A279T	JP 1994062866-A	E06826
1,50 20020		(sequence 2)	
lysCFBR-E06827		JP 1994062866-A	E06827
		(sequence 3)	
lysCFER-E08177		JP 1994261766-A	E08177
		(sequence 1)	
lysCFBR-E08178	lysC A279T	JP 1994261766-A	E08178
_	_	(sequence 2)	
lysCFBR-E08179	lysC A279V	JP 1994261766-A	E08179
_		(sequence 3)	
lysCFBR-E08180	lysC S301F	JP 1994261766-A	E08180
		(sequence 4)	
lysCFBR-E08181	lysC T308I	JP 1994261766-A	E08181
		(sequence .5)	
lysCFBR-E08182		JP 1994261766-A	E08182
		(sequence 6)	
lysCFBR-E12770		JP 1997070291-A	E12770
		(sequence 13)	714514
lysCFBR-E14514		JP 1997322774-A	E14514
EDD.		(sequence 9)	m1.C2.E.2
lysC ^{FBR} -E16352		JP 1998165180-A	E16352
FBD - 4 FF 4 F		(sequence 3)	E16745
lysC ^{FBR} -E16745		JP 1998215883-A	F10142
- FBR - CTAC		(sequence 3) JP 1998215883-A	E16746
lysCFER-E16746)	ET0140
1 0FBR 774500		US 5688671-A	I74588
lysC ^{FBR} -174588		(sequence 1)	174300
1CFBR T74500	1C 3270m	US 5688671-A	174589
lysC ^{FBR} -174589	lysC A279T	(sequence 2)	11,200
7 OFAR T74500		US 5688671-A	174590
lysCFBR-I74590		(sequence 7)	1,4330
lysC ^{FBR} -174591	lysC A279T	US 5688671-A	174591
TAPCT.#131	TASC BELLI	(sequence 8)	
lysC ^{FBR} -I74592		US 5688671-A	174592
TASC -114225		(sequence 9)	
lysC ^{FBR} -I74593	lysC A279T	US 5688671-A	174593
TADO TIEDO		(sequence 10)	
lysC ^{FBR} -174594		US 5688671-A	174594
		(sequence 11)	
1ysCFBR-174595	lysC A279T	US 5688671-A	174595
		(sequence 12)	
1ysCFBR-174596		US 5688671-A	174596
		(sequence 13)	· .
			

lysCFBR-I74597	13xcC 3270m	TO FORCER >	
T320 -114331	lysC A279T	US 5688671-A	174597
lysCFBR-X57226	1	(sequence 14)	
TARC -X21776	lysC S301Y	EP0387527	X57226
	j	Kalinowski et al.,	
		Molecular and	Í
		General Genetics	
7 FRP		224:317-324 (1990)	
lysCFBR-L16848	lysc G345D	Follettie and	L16848
	•	Sinskey	
		NCBI Nucleotide	
7 500		Database (1990)	
lysC ^{FBR} -L27125	lysC R320G	Jetten et al.,	L27125
	lysC G345D	Applied Microbiology	
		Biotechnology 43:76-	·
		82 (1995)	
lysCFBR	lysC T311I	WO0063388	
		(sequence 17)	
lysCFBR	lysC S301F	US3732144	
1ysC ^{FBR}]vrcC C201E		
-300	lysC S381F		
lysCFBR		JP6261766	
		(sequence 1)	
lysCFBR	lysc A279T	JP6261766	·
		(sequence 2)	
lysCFBR	lysC A279V	JP6261766	
		(sequence 3)	
lysCFBR	lysC S301F	JP6261766	
		(sequence 4)	
1ysC ^{FBR}	1ysC T308I	JP6261766	
		(sequence 5)	

Table 3

Target sites for integration of open reading frames, genes and alleles of lysine production

Gene	Description of the coded	Reference	Access
name	enzyme or protein		Number
			1.000
aecD	beta C-S Lyase	Rossol et al., Journal	M89931
	EC 2.6.1.1	of Bacteriology	
	(beta C-S lyase)	174(9):2968-77 (1992)	
ccpA1	Catabolite Control	WO0100844	AX065267
	Protein	EP1108790	AX127147
	(catabolite control		
	protein A1)		·
ccpA2	Catabolite Control	WO0100844	AX065267
	Protein	EP1108790	AX121594
	(catabolite control		
	protein A2)		
citA	Sensor Kinase CitA	EP1108790	AX120161
	(sensor kinase CitA)	• •	
citB	Transcription Regulator	EP1108790	AX120163
	CitB		
	(transcription regulator	•	·
	CitB)		
citE	Citrate Lyase	WO0100844	AX065421
	EC 4.1.3.6	EP1108790	AX127146
	(citrate lyase)		
fda	Fructose Bisphosphate	von der Osten et al.,	X17313
	Aldolase	Molecular Microbiology	
	EC 4.1.2.13	3(11):1625~37 (1989)	
	(fructose 1,6-		
	bisphosphate aldolase)		
gluA	Glutamate Transport ATP-	Kronemeyer et al.,	X81191
	binding Protein	Journal of Bacteriology	
	(glutamate transport	177(5):1152-8 (1995)	
	ATP-binding protein)		
gluB	Glutamate-binding	Kronemeyer et al.,	X81191
	Protein	Journal of Bacteriology	
	(glutamate-binding	177(5):1152-8 (1995)	}
	protein)		
gluC	Glutamate Transport	Kronemeyer et al.,	X81191
	Permease	Journal of Bacteriology	
	(glutamate transport	177(5):1152-8 (1995)	
-1D	system permease)		
gluD	Glutamate Transport	Kronemeyer et al.,	X81191
	Permease	Journal of Bacteriology	
	(glutamate transport	177(5):1152-8 (1995)]
7	system permease)		777065050
luxR	Transcription Regulator	WO0100842	AX065953
	LuxR	EP1108790	AX123320
	(transcription regulator		
7,,,,,,,	LuxR)	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	3714 022 02
luxS	Histidine Kinase LuxS	EP1108790	AX123323
7	(histidine kinase LuxS)		AX127145
lysR1	Transcription Regulator	EP1108790	AX064673
	LysR1		AX127144

	(transcription regulator LysR1)		
lysR2	Transcription Activator LysR2	· EP1108790	AX123312
	(transcription regulator LysR2)	·	
lysR3	Transcription Regulator	WO0100842	AX065957
	LysR3	EP1108790	AX127150
	(transcription regulator LysR3)		
menE	O-Succinylbenzoic Acid	WO0100843	AX064599
	CoA Ligase	EP1108790	AX064193
	EC 6.2.1.26		AX127144
	(O-succinylbenzoate CoA		
	ligase)		
mgo	Malate-Quinone	Molenaar et al., Eur.	AJ224946
	Oxidoreductase	Journal of Biochemistry	
	(malate-quinone-	1;254(2):395-403 (1998)	
	oxidoreductase)		
pck	Phosphoenol Pyruvate	WO0100844	AJ269506
i	Carboxykinase		AX065053
	(phosphoenol pyruvate		
	carboxykinase)		•
pgi	Glucose 6-phosphate	EP1087015	AX136015
	Isomerase	EP1108790	AX127146
	EC 5.3.1.9		
	(glucose 6-phosphate		
	isomerase)		
рохВ	Pyruvate Oxidase	WO0100844	AX064959
	EC 1.2.3.3	EP1096013	AX137665
	(pyruvate oxidase)	<u> </u>	
zwa2	Cell Growth Factor 2	EP1106693	AX113822
	(growth factor 2)	EP1108790	AX127146

The invention accordingly also provides a process for the production of coryneform bacteria which produce L-lysine, which comprises

- a) isolating the nucleotide sequence of at least one desired ORF, gene or allele of lysine production, optionally including the expression and/or regulation signals,
- 10 b) providing the 5' and the 3' end of the ORF, gene or allele of lysine production with nucleotide sequences of the target site,
 - c) preferably incorporating the nucleotide sequence of the desired ORF, gene or allele provided with

nucleotide sequences of the target site into a vector which does not replicate or replicates to only a limited extent in coryneform bacteria,

d) transferring the nucleotide sequence according to b) or c) into coryneform bacteria, and

5

10

e) isolating coryneform bacteria in which the nucleotide sequence according to a) is incorporated at the target site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the target site.

The invention furthermore provides coryneform bacteria, in particular of the genus Corynebacterium, which produce L-15 methionine and/or L-threonine, characterized in that these have, in addition to at least one of the copy of an open reading frame (ORF), gene or allele of methionine production or threonine production present at the natural site (locus), in each case a second, optionally third or fourth copy of the open reading frame (ORF), gene or allele in question at in each case a second, optionally third or fourth site in integrated form, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable 25 of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular second, optionally third or fourth site.

The invention also furthermore provides a process for the preparation of L-methionine and/or L-threonine, which comprises the following steps:

a) fermentation of coryneform bacteria, in particular Corynebacterium glutamicum, characterized in that

these have, in addition to at least one of the copy of an open reading frame (ORF), gene or allele of methionine production or threonine production present at the natural site (locus), in each case a second, optionally third or fourth copy of the open reading frame (ORF), gene or allele in question at in each case a second, optionally third or fourth site in integrated form, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular second, optionally third or fourth site,

PCT/EP02/08464

15

10

5

- under conditions which allow expression of the said open reading frames (ORF), genes or alleles,
- b) concentration of the L-methionine and/or L-threonine in the fermentation broth,
- 20 c) isolation of the L-methionine and/or L-threonine from the fermentation broth, optionally
 - d) with constituents from the fermentation broth and/or the biomass to the extent of > (greater than) 0 to 100%.
- A "copy of an open reading frame (ORF), gene or allele of methionine production" is to be understood as meaning all the, preferably endogenous, open reading frames, genes or alleles of which enhancement/over-expression can have the effect of improving methionine production.
- These include, inter alia, the following open reading frames, genes or alleles: accBC, accDA, aecD, cstA, cysD, cysE, cysH, cysK, cysN, cysQ, dps, eno, fda, gap, gap2, gdh, gnd, glyA, hom, hom^{FBR}, lysC, lysC^{FBR}, metA, metB, metE,

metH, metY, msiK, opcA, oxyR, ppc, ppc^{FBR}, pgk, pknA, pknB, pknD, pknG, ppsA, ptsH, ptsI, ptsM, pyc, pyc P458S, sigC, sigD, sigE, sigH, sigM, tal, thyA, tkt, tpi, zwa1, zwf and zwf A213T. These are summarized and explained in Table 4. These include, in particular, the lysC^{FBR} alleles which code for a "feed back" resistant aspartate kinase (see Table 2) and the hom^{FBR} alleles which code for a "feed back" resistant homoserine dehydrogenase.

The second, optionally third or fourth copy of the open reading frame (ORF), gene or allele of methionine production in question can be integrated at in each case a second, optionally third or fourth site. The following open reading frames, genes or nucleotide sequences, inter alia, can be used for this: brnE, brnF, brnQ, ccpA1, ccpA2, citA, citB, citE, ddh, gluA, gluB, gluC, gluD, luxR, luxS, lysR1, lysR2, lysR3, menE, metD, metK, pck, pgi, poxB and zwa2. These are summarized and explained in Table 5.

The sites mentioned include, of course, not only the coding regions of the open reading frames or genes mentioned, but also the regions or nucleotide sequences lying upstream 20 which are responsible for expression and regulation, such as, for example, ribosome binding sites, promoters, binding sites for regulatory proteins, binding sites for regulatory ribonucleic acids and attenuators. These regions in general lie in a range of 1-800, 1-600, 1-400, 1-200, 1-100 or 1-50 25 nucleotides upstream of the coding region. In the same way, regions lying downstream, such as, for example, transcription terminators, are also included. These regions in general lie in a range of 1-400, 1-200, 1-100, 1-50 or 1-25 nucleotides downstream of the coding region. 30

Intergenic regions in the chromosome, that is to say nucleotide sequences without a coding function, can furthermore be used. Finally, prophages or defective phages contained in the chromosome can be used for this.

PCT/EP02/08464 WO 03/040373 28

Table 4 Open reading frames, genes and alleles of methionine production

Name	Description of the coded enzyme or	Reference	Access
	protein		Number
AccBC	Acyl-CoA Carboxylase	Jäger et al.	U35023
	EC 6.3.4.14	Archives of	
	(acyl-CoA carboxylase)	Microbiology	
}		(1996) 166:76-82	
		EP1108790;	AX123524
ļ		W00100805	AX066441
AccDA	Acetyl-CoA Carboxylase	EP1055725	
	EC 6.4.1.2	EP1108790	AX121013
	(acetyl-CoA carboxylase)	W00100805	AX066443
AecD	Cystathionine beta-Lyase	Rossol et al.,	M89931
	EC 4.4.1.8	Journal of	İ
	(cystathionine beta-lyase)	Bacteriology	
		174:2968-2977	
		(1992)	
CstA	Carbon Starvation Protein A	EP1108790	AX120811
	(carbon starvation protein A)	WO0100804	AX066109
CysD	Sulfate Adenylyltransferase	EP1108790	AX123177
_	sub-unit II	1	
	EC 2.7.7.4	}	}
	(sulfate adenylyltransferase small	}	
	chain)		
CysE	Serine Acetyltransferase	EP1108790	AX122902
	EC 2.3.1.30	WO0100843	AX063961
	(serine acetyltransferase)		
CysH	3'-Phosphoadenyl Sulfate Reductase	EP1108790	AX123178
	EC 1.8.99.4	WO0100842	AX066001
	(3'-phosphoadenosine 5'-		
	phosphosulfate reductase)	<u> </u>	
CysK	Cysteine Synthase	EP1108790	AX122901
	EC 4.2.99.8	WO0100843	AX063963
	(cysteine synthase)		
CysN	Sulfate Adenylyltransferase sub-	EP1108790	AX123176
	unit I	<u> </u>	AX127152
	EC 2.7.7.4	<u>}</u>	
 	(sulfate adenylyltransferase)		
CysQ	Transport protein CysQ	EP1108790	AX127145
	(transporter cysQ)	WO0100805	AX066423
Dps	DNA Protection Protein	EP1108790	AX127153
	(protection during starvation	\$	
	protein)	<u> </u>	
Eno	Enolase	EP1108790	AX127146
	EC 4.2.1.11	WO0100844	AX064945
	(enolase)	EP1090998	AX136862
	·	Hermann et al.,	
		Electrophoresis	
		19:3217-3221	
		(1998)	

Fda	Fructose Bisphosphate Aldolase EC 4.1.2.13 (fructose bisphosphate aldolase)	van der Osten et al., Molecular Microbiology	X17313
		3:1625-1637 (1989)	
Gap	Glyceraldehyde 3-Phosphate Dehydrogenase EC 1.2.1.12	EP1108790 WO0100844	AX127148 AX064941
	(glyceraldehyde 3-phosphate dehydrogenase)	Eikmanns et al., Journal of Bacteriology	x59403
		174:6076- 6086(1992)	
gap2	Glyceraldehyde 3-Phosphate Dehydrogenase EC 1.2.1.12 (glyceraldehyde 3-phosphate	EP1108790 WO0100844	AX127146 AX064939
	dehydrogenase 2)		
Gđh	Glutamate Dehydrogenase EC 1.4.1.4	EP1108790 WO0100844	AX127150 AX063811
	(glutamate dehydrogenase)	Boermann et al., Molecular Microbiology 6:317-326	X59404
		(1992);	
		Guyonvarch et al., NCBI	X72855
GlyA	Glycine/Serine Hydroxymethyltransferase	EP1108790	AX127146 AX121194
	EC 2.1.2.1		AVISILA
	(glycine/serine hydroxymethyltransferase)		
Gnd	6-Phosphogluconate Dehydrogenase	EP1108790	AX127147
	EC 1.1.1.44		AX121689
	(6-phosphogluconate dehydrogenase)	WO0100844	AX065125
Hom	Homoserine Dehydrogenase EC 1.1.1.3	Peoples et al., Molecular	Y00546
	(homoserine dehydrogenase)	Microbiology	
hom ^{FBR}	Homoserine Dehydrogenase feedback	2:63-72 (1988) Reinscheid et	
110/11	resistant (fbr)	al., Journal of	
	EC 1.1.1.3	Bacteriology	
	(homoserine dehydrogenase fbr)	173:3228-30 (1991)	
LysC	Aspartate Kinase	EP1108790	AX120365
	EC 2.7.2.4	WO0100844	AX063743
	(aspartate kinase)	Kalinowski et al., Molecular	X57226
		Microbiology	
		5:1197-204 (1991)	
lysCFBR	Aspartate Kinase feedback resistant	see Table 2	
	(fbr) EC 2.7.2.4		
34-43	(aspartate kinase fbr)		
MetA	Homoserine Acetyltransferase EC 2.3.1.31	Park et al., Molecular Cells	AF052652
	(homoserine acetyltransferase)	8:286-94 (1998)	
MetB	Cystathionine γ-Lyase	Hwang et al.,	AF126953

		Malagalan Calla	
	EC 4.4.1.1	Molecular Cells	
	(cystathionine gamma-synthase)	9:300-308 (1999)	AX127146
MetE	Homocysteine Methyltransferase	EP1108790	
	EC 2.1.1.14		AX121345
	(homocysteine methyltransferase)	<u> </u>	
MetH	Homocysteine Methyltransferase	EP1108790	AX127148
	(Vitamin B12-dependent)		AX121747
	EC 2.1.1.14		
	(homocysteine methyltransferase)		
MetY	Acetylhomoserine Sulfhydrolase	EP1108790	AX120810
	(acetylhomoserine sulfhydrolase)		AX127145
MsiK	Sugar Importer	EP1108790	AX120892
	(multiple sugar import protein)		
OpcA	Glucose 6-phosphate Dehydrogenase	WO0104325	AX076272
	(subunit of glucose 6-phosphate		
	dehydrogenase)		
OxyR	Transcription Regulator	EP1108790	AX122198
	(transcriptional regulator)		AX127149
ppcFBR	Phosphoenol Pyruvate Carboxylase	EP0723011	
	feedback resistent	WO0100852	
	EC 4.1.1.31		·
	(phosphoenol pyruvate carboxylase		
	feedback resistant)		
Ppc	Phosphoenol Pyruvate Carboxylase	EP1108790	AX127148
-	EC 4.1.1.31		AX123554
	(phosphoenol pyruvate carboxylase)	O'Reagan et al.,	M25819
		Gene 77(2):237-	
•		251 (1989)	
Pgk	Phosphoglycerate Kinase	EP1108790	AX121838
_	EC 2.7.2.3		AX127148
	(phosphoglycerate kinase)	WO0100844	AX064943
		Eikmanns,	X59403
		Journal of	
		Bacteriology	
		174:6076-6086	Ì
		(1992)	
PknA	Protein Kinase A	EP1108790	AX120131
	(protein kinase A)		AX120085
PknB	Protein Kinase B	EP1108790	AX120130
	(protein kinase B)		AX120085
PknD	Protein Kinase D	EP1108790	AX127150
	(protein Kinase D)	}	AX122469
······································			AX122468
PknG	Protein Kinase G	EP1108790	AX127152
	(protein kinase G)		AX123109
PpsA	Phosphoenol Pyruvate Synthase	EP1108790	AX127144
	EC 2.7.9.2		AX120700
		l	AX122469
	(phosphoenol pyruvate synthase)		
PtsH	Phosphotransferase System Protein H	EP1108790	AX122210
PtsH			AX127149
PtsH	Phosphotransferase System Protein H	EP1108790 WO0100844	1
PtsH	Phosphotransferase System Protein H EC 2.7.1.69 (phosphotransferase system component H)	WO0100844	AX127149 AX069154
PtsH PtsI	Phosphotransferase System Protein H EC 2.7.1.69 (phosphotransferase system		AX127149 AX069154 AX122206
•	Phosphotransferase System Protein H EC 2.7.1.69 (phosphotransferase system component H)	WO0100844	AX127149 AX069154
•	Phosphotransferase System Protein H EC 2.7.1.69 (phosphotransferase system component H) Phosphotransferase System Enzyme I	WO0100844	AX127149 AX069154 AX122206
•	Phosphotransferase System Protein H EC 2.7.1.69 (phosphotransferase system component H) Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I)	WO0100844 EP1108790	AX127149 AX069154 AX122206 AX127149
•	Phosphotransferase System Protein H EC 2.7.1.69 (phosphotransferase system component H) Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system	WO0100844 EP1108790	AX127149 AX069154 AX122206 AX127149

	FC 2 7 1 60		
}	EC 2.7.1.69	Letters 119	
1	(glucose phosphotransferase system	(1-2):137-145	
	enzyme II)	(1994)	
Рус	Pyruvate Carboxylase	W09918228	A97276
	EC 6.4.1.1	Peters-Wendisch	Y09548
	(pyruvate carboxylase)	et al.,	
		Microbiology	
		144:915-927	
		(1998)	
Рус	Pyruvate Carboxylase	EP1108790	
P458s	EC 6.4.1.1	1311100730	
	(pyruvate carboxylase)	1	
	amino acid exchange P458S		
SigC	Sigma Factor C	EP1108790	24120260
Dige	EC 2.7.7.6	EP1100/90	AX120368
	(extracytoplasmic function	1	AX120085
	alternative sigma factor C)		
SigD	RNA Polymerase Sigma Factor D	TD1100000	
Digb	EC 2.7.7.6	EP1108790	AX120753
		1	AX127144
SigE	(RNA polymerase sigma factor)		
Sign	Sigma Factor E EC 2.7.7.6	EP1108790	AX127146
		·	AX121325
	(extracytoplasmic function		
Citari	alternative sigma factor E)		
SigH	Sigma Factor H	EP1108790	AX127145
	EC 2.7.7.6		AX120939
G: -35	(sigma factor SigH)		
SigM	Sigma Factor M	EP1108790	AX123500
ļ	EC 2.7.7.6		AX127153
	(sigma factor SigM)		
Tal	Transaldolase	WO0104325	AX076272
	EC 2.2.1.2		
	(transaldolase)		
ThyA	Thymidylate Synthase	EP1108790	AX121026
	EC 2.1.1.45		AX127145
	(thymidylate synthase)		
Tkt	Transketolase	Ikeda et al.,	AB023377
	EC 2.2.1.1	NCBI	
	(transktolase)	}	
Tpi	Triose Phosphate Isomerase	Eikmanns,	X59403
	EC 5.3.1.1	Journal of	
	(triose phosphate isomerase)	Bacteriology	
		174:6076-6086	
		(1992)	,
zwa1	Cell Growth Factor 1	EP1111062	AX133781
	(growth factor 1)		
Zwf	Glucose 6-phosphate 1-Dehydrogenase	EP1108790	AX127148
	EC 1.1.1.49		AX121827
	(glucose 6-phosphate 1-	W00104325	AX076272
	dehydrogenase)	110020200	ANUIUZIZ
Zwf	Glucose 6-phosphate 1-Dehydrogenase	EP1108790	
A213T	EC 1.1.1.49	ELTTO120	
W2T2T	(glucose 6-phosphate 1-		
	dehydrogenase)		1
	amino acid exchange A213T		
	mittio actu excitatige AZIST	<u> </u>	<u></u>

Table 5

Target sites for integration of open reading frames, genes and alleles of methionine production

Gene	Description of the	Reference	Access
	coded enzyme or protein	Ref Cr Chico	Number
name	Coded enzyme or processi	·	
BrnE	Transporter of	EP1096010	AX137709
DLIE	branched-chain amino	211030010	AX137714
	acids		
	(branched-chain amino		
	acid transporter)		
BrnF	Transporter of	EP1096010	AX137709
DTIIL	branched-chain amino	111030010	AX137714
	acids		
	(branched-chain amino		
	acid transporter)		
PrnO	Carrier protein of	Tauch et al., Archives	M89931
BrnQ	branched-chain amino	of Microbiology	AX066841
	acids	169(4):303-12 (1998)	AX127150
	(branched-chain amino	WO0100805	
	acid transport system	EP1108790	1
	carrier protein)		
ccpA1	Catabolite Control	WO0100844	AX065267
CCDAI	Protein	EP1108790	AX127147
	(catabolite control		
	protein A1)		
ccpA2	Catabolite Control	WQ0100844	AX065267
Copina	Protein	EP1108790	AX121594
	(catabolite control		
	protein A2)		
citA	Sensor Kinase CitA	EP1108790	AX120161
	(sensor kinase CitA)		
citB	Transcription Regulator	EP1108790	AX120163
	CitB		
	(transcription		
	regulator CitB)		
citE	Citrate Lyase	WO0100844	AX065421
	EC 4.1.3.6	EP1108790	AX127146
	(citrate lyase)		•
ddh	Diaminopimelate	Ishino et al., Nucleic	S07384
	Dehydrogenase	Acids Research 15: 3917	AX127152
	EC 1.4.1.16	(1987)	
	(diaminopimelate	EP1108790	
	dehydrogenase)		
gluA	Glutamate Transport	Kronemeyer et al.,	x81191
	ATP-binding Protein	Journal of Bacteriology	
	(glutamate transport	177(5):1152-8 (1995)	
	ATP-binding protein)		******
gluB	Glutamate-binding	Kronemeyer et al.,	X81191
	Protein	Journal of Bacteriology	
	(glutamate-binding	177(5):1152-8 (1995)	
	protein)		********
gluC	Glutamate Transport	Kronemeyer et al.,	X81191
	Permease	Journal of Bacteriology	
	(glutamate transport	177(5):1152-8 (1995)	

	system permease)		<u> </u>
gluD	Glutamate Transport	Kronemeyer et al.,	X81191
	Permease	Journal of Bacteriology	VOTIAT
	(glutamate transport	177(5):1152-8 (1995)	
	system permease)	177(37.1132"0 (1993)	
luxR	Transcription Regulator	WO0100842	AV065052
	LuxR	EP1108790	AX065953
	(transcription	EPIIO0790	AX123320
	regulator LuxR)		
luxS	Histidine Kinase LuxS	EP1108790	224 02 202
	(histidine kinase LuxS)	EPITUO/90	AX123323
lysR1	Transcription Regulator	ED1100700	AX127145
-, D	LysR1	EP1108790	AX064673
	(transcription		AX127144
	regulator LysR1)		
lysR2	Transcription Activator	FD1100700	2224 0 0 0 0 0
470112	LysR2	EP1108790	AX123312
	(transcription		•
	regulator LysR2)		
lysR3	Transcription Regulator	W00100042	311065055
	LysR3	WO0100842	AX065957
	(transcription	EP1108790	AX127150
	regulator LysR3)		
menE	O-Succinylbenzoic Acid	W00100043	2****
-110362	CoA Ligase	WO0100843	AX064599
	EC 6.2.1.26	EP1108790	AX064193
	(O-succinylbenzoate CoA	_	AX127144
	ligase)		
metD	Transcription Regulator	EP1108790	AX123327
	MetD	E1 1100790	AX127153
	(transcription		HVT7/T77
	regulator MetD)	j	
metK	Methionine Adenosyl	WO0100843	AX063959
	Transferase	EP1108790	AX127148
	EC 2.5.1.6		MILZ, ILTO
	(S-adenosylmethionine	i	
	synthetase)		
pck	Phosphoenol Pyruvate	WO0100844	AJ269506
	Carboxykinase		AX065053
	(phosphoenol pyruvate		12.005055
	carboxykinase)	<u>-</u>	·
pgi	Glucose 6-Phosphate	EP1087015	AX136015
	Isomerase	EP1108790	AX127146
	EC 5.3.1.9		
ĺ	(glucose-6-phosphate		j
	isomerase)		
рохВ	Pyruvate Oxidase	WO0100844	AX064959
	EC 1.2.3.3	EP1096013	AX137665
	(pyruvate oxidase)		
zwa2	Cell Growth Factor 2	EP1106693	AX113822
	(growth factor 2)	EP1108790	AX127146

A "copy of an open reading frame (ORF), gene or allele of threonine production" is to be understood as meaning all the open reading frames, genes or alleles of which enhancement/over-expression can have the effect of improving threonine production.

These include, inter alia, the following open reading frames, genes or alleles: accBC, accDA, cstA, cysD, cysE, cysH, cysI, cysN, cysQ, dps, eno, fda, gap, gap2, gdh, gnd, hom, hom^{FBR}, lysC, lysC^{FBR}, msiK, opcA, oxyR, ppc, ppc^{FBR}, pgk, pknA, pknB, pknD, pknG, ppsA, ptsH, ptsI, ptsM, pyc, pyc P458S, sigC, sigD, sigE, sigH, sigM, tal, thyA, tkt, tpi, thrB, thrC, thrE, zwal, zwf and zwf A213T. These are summarized and explained in Table 6. These include, in particular, the lysC^{FBR} alleles which code for a "feed back" resistant aspartate kinase (See Table 2) and the hom^{FBR} alleles which code for a "feed back" resistant homoserine dehydrogenase.

The second, optionally third or fourth copy of the open reading frame (ORF), gene or allele of threonine production in question can be integrated at in each case a second, optionally third or fourth site. The following open reading frames, genes or nucleotide sequences, inter alia, can be used for this: ccpA1, ccpA2, citA, citB, citE, ddh, gluA, gluB, gluC, gluD, glyA, ilvA, ilvBN, ilvC, ilvD, luxR, luxS, lysR1, lysR2, lysR3, mdh, menE, metA, metD, pck, poxB, sigB and zwa2. These are summarized and explained in Table 7.

The sites mentioned include, of course, not only the coding regions of the open reading frames or genes mentioned, but also the regions or nucleotide sequences lying upstream which are responsible for expression and regulation, such as, for example, ribosome binding sites, promoters, binding sites for regulatory proteins, binding sites for regulatory ribonucleic acids and attenuators. These regions in general lie in a range of 1-800, 1-600, 1-400, 1-200, 1-100 or 1-50

nucleotides upstream of the coding region. In the same way, regions lying downstream, such as, for example, transcription terminators, are also included. These regions in general lie in a range of 1-400, 1-200, 1-100, 1-50 or 1-25 nucleotides downstream of the coding region.

35

Intergenic regions in the chromosome, that is to say nucleotide sequences without a coding function, can furthermore be used. Finally, prophages or defective phages contained in the chromosome can be used for this.

Table 6

Open reading frames, genes and alleles of threonine production

Name	Description of the coded enzyme or	Reference	Access
	protein		Number
Í			
accBC	Acyl-CoA Carboxylase	Jäger et al.	U35023
}	EC 6.3.4.14	Archives of	
	(acyl-CoA carboxylase)	Microbiology	
	,	166:76-82 (1996)	
		EP1108790	AX123524
1	1	W00100805	AX066441
accDA	Acetyl-CoA Carboxylase	EP1055725	121000357
	EC 6.4.1.2	EP1108790	AX121013
ţ	(acetyl-CoA carboxylase)	WO0100805	AX066443
cstA	Carbon Starvation Protein A	EP1108790	AX120811
	(carbon starvation protein A)	WO0100804	AX066109
cysD	Sulfate Adenylyltransferase	EP1108790	AX123177
Cysb	sub-unit II	EP1108/90	AX1231//
	EC 2.7.7.4	1	
	(sulfate adenylyltransferase small		
	chain)		
Greek		PD1100700	77100000
cysE	Serine Acetyltransferase EC 2.3.1.30	EP1108790	AX122902
	(serine acetyltransferase)	WO0100843	AX063961
Credi		ED1100700	77102170
cysH	3'-Phosphoadenyl Sulfate Reductase EC 1.8.99.4	EP1108790	AX123178
{	(3'-phosphoadenosine 5'-	WO0100842	AX066001
	phosphosulfate reductase)		
cysK	Cysteine Synthase	EP1108790	37122001
Cysk	EC 4.2.99.8	WO0100843	AX122901 AX063963
	(cysteine synthase)	WOOTOO843	AAUG39G3
cysN	Sulfate Adenylyltransferase sub-	EP1108790	AX123176
CASM	unit I	EP1108/90	AX123176 AX127152
	EC 2.7.7.4	Ì	HAIZ/152
	(sulfate adenylyltransferase)		
arra0		ED1100700	37107145
cysQ	Transport protein CysQ	EP1108790	AX127145
2	(transporter cysQ) DNA Protection Protein	WO0100805	AX066423
dps	1	EP1108790	AX127153
	(protection during starvation	i	
	protein)	771100700	37107116
eno	Enolase EC 4.2.1.11	EP1108790	AX127146
		WO0100844	AX064945
	(enolase)	EP1090998	AX136862
		Hermann et al.,	
		Electrophoresis	
		19:3217-3221	[
£ 3_	Emistone Disphase 33-3-1	(1998)	77 77 77
fda	Fructose Bisphosphate Aldolase	van der Osten et	X17313
	EC 4.1.2.13	al., Molecular	1
	(fructose bisphosphate aldolase)	Microbiology	
		3:1625-1637	
	Olympia de la companya de la company	(1989)	2000000
gap	Glyceraldehyde 3-Phosphate	EP1108790	AX127148
	Dehydrogenase	WO0100844	AX064941
	EC 1.2.1.12	Eikmanns et al.,	X59403

	(almoss 1 dob		
	(glyceraldehyde 3-phosphate	Journal of	
	dehydrogenase)	Bacteriology	
		174:6076-	
		6086 (1992)	
gap2	Glyceraldehyde 3-Phosphate	EP1108790	AX127146
	Dehydrogenase	WO0100844	AX064939
	EC 1.2.1.12		
	(glyceraldehyde 3-phosphate	-	
	dehydrogenase 2)		
gdh	Glutamate Dehydrogenase	EP1108790	AX127150
	EC 1.4.1.4	WO0100844	AX063811
	(glutamate dehydrogenase)	Boermann et al.,	X59404
		Molecular	
		Microbiology	
		6:317-326	
		(1992);	
		Guyonvarch et	X72855
		al., NCBI	12033
gnd	6-Phosphogluconate Dehydrogenase	EP1108790	AX127147
	EC 1.1.1.44		AX121689
	(6-phosphogluconate dehydrogenase)	WO0100844	AX065125
hom	Homoserine Dehydrogenase	Peoples et al.,	Y00546
	EC 1.1.1.3	Molecular	100240
	(homoserine dehydrogenase)	Microbiology	ļ
·	(1101110 delly delogellase)	2:63-72 (1988)	
homFBR	Homoserine Dehydrogenase feedback	Reinscheid et	
	resistant (fbr)	1	
	EC 1.1.1.3	al., Journal of	
	(homoserine dehydrogenase fbr)	Bacteriology	
	(nomoser the denydrogenase IDI)	(1991)	
lysC	Aspartate Kinase	EP1108790	3V120265
1300	EC 2.7.2.4	WO0100844	AX120365
	(aspartate kinase)		AX063743
	(asparcace kinase)	Kalinowski et	X57226
		al., Molecular	
		Microbiology	}
		5:1197-204 (1991)	
lysCPBR	Aspartate Kinase feedback resistent	<u> </u>	
	(fbr)	see rable 2	
	EC 2.7.2.4	i	
			1
	(aspartate kinace fbr)		[
meik	(aspartate kinase fbr) Sugar Importer	ED1100700	AV120002
msiK	Sugar Importer	EP1108790	AX120892
	Sugar Importer (multiple sugar import protein)		
msiK opcA	Sugar Importer (multiple sugar import protein) Glucose 6-Phosphate Dehydrogenase	EP1108790 WO0104325	AX120892 AX076272
	Sugar Importer (multiple sugar import protein) Glucose 6-Phosphate Dehydrogenase (subunit of glucose 6-phosphate		
opcA	Sugar Importer (multiple sugar import protein) Glucose 6-Phosphate Dehydrogenase (subunit of glucose 6-phosphate dehydrogenase)	WO0104325	AX076272
opcA	Sugar Importer (multiple sugar import protein) Glucose 6-Phosphate Dehydrogenase (subunit of glucose 6-phosphate dehydrogenase) Transcription Regulator		AX076272 AX122198
орсА охуR	Sugar Importer (multiple sugar import protein) Glucose 6-Phosphate Dehydrogenase (subunit of glucose 6-phosphate dehydrogenase) Transcription Regulator (transcriptional regulator)	WO0104325 EP1108790	AX076272
opcA	Sugar Importer (multiple sugar import protein) Glucose 6-Phosphate Dehydrogenase (subunit of glucose 6-phosphate dehydrogenase) Transcription Regulator (transcriptional regulator) Phosphoenol Pyruvate Carboxylase	WO0104325 EP1108790 EP0723011	AX076272 AX122198
орсА охуR	Sugar Importer (multiple sugar import protein) Glucose 6-Phosphate Dehydrogenase (subunit of glucose 6-phosphate dehydrogenase) Transcription Regulator (transcriptional regulator) Phosphoenol Pyruvate Carboxylase feedback resistent	WO0104325 EP1108790	AX076272 AX122198
орсА охуR	Sugar Importer (multiple sugar import protein) Glucose 6-Phosphate Dehydrogenase (subunit of glucose 6-phosphate dehydrogenase) Transcription Regulator (transcriptional regulator) Phosphoenol Pyruvate Carboxylase feedback resistent EC 4.1.1.31	WO0104325 EP1108790 EP0723011	AX076272 AX122198
орсА охуR	Sugar Importer (multiple sugar import protein) Glucose 6-Phosphate Dehydrogenase (subunit of glucose 6-phosphate dehydrogenase) Transcription Regulator (transcriptional regulator) Phosphoenol Pyruvate Carboxylase feedback resistent EC 4.1.1.31 (phosphoenol pyruvate carboxylase	WO0104325 EP1108790 EP0723011	AX076272 AX122198
opcA oxyR ppc ^{FER}	Sugar Importer (multiple sugar import protein) Glucose 6-Phosphate Dehydrogenase (subunit of glucose 6-phosphate dehydrogenase) Transcription Regulator (transcriptional regulator) Phosphoenol Pyruvate Carboxylase feedback resistent EC 4.1.1.31 (phosphoenol pyruvate carboxylase feedback resistant)	WO0104325 EP1108790 EP0723011 WO0100852	AX122198 AX127149
opcA oxyR ppcFER	Sugar Importer (multiple sugar import protein) Glucose 6-Phosphate Dehydrogenase (subunit of glucose 6-phosphate dehydrogenase) Transcription Regulator (transcriptional regulator) Phosphoenol Pyruvate Carboxylase feedback resistent EC 4.1.1.31 (phosphoenol pyruvate carboxylase feedback resistant) Phosphoenol Pyruvate Carboxylase	WO0104325 EP1108790 EP0723011	AX122198 AX127149
opcA oxyR ppc ^{FBR}	Sugar Importer (multiple sugar import protein) Glucose 6-Phosphate Dehydrogenase (subunit of glucose 6-phosphate dehydrogenase) Transcription Regulator (transcriptional regulator) Phosphoenol Pyruvate Carboxylase feedback resistent EC 4.1.1.31 (phosphoenol pyruvate carboxylase feedback resistant) Phosphoenol Pyruvate Carboxylase feedback resistant)	WO0104325 EP1108790 EP0723011 WO0100852	AX122198 AX127149
opcA oxyR ppcFBR	Sugar Importer (multiple sugar import protein) Glucose 6-Phosphate Dehydrogenase (subunit of glucose 6-phosphate dehydrogenase) Transcription Regulator (transcriptional regulator) Phosphoenol Pyruvate Carboxylase feedback resistent EC 4.1.1.31 (phosphoenol pyruvate carboxylase feedback resistant) Phosphoenol Pyruvate Carboxylase	WO0104325 EP1108790 EP0723011 WO0100852	AX122198 AX127149
opcA oxyR ppc ^{FBR}	Sugar Importer (multiple sugar import protein) Glucose 6-Phosphate Dehydrogenase (subunit of glucose 6-phosphate dehydrogenase) Transcription Regulator (transcriptional regulator) Phosphoenol Pyruvate Carboxylase feedback resistent EC 4.1.1.31 (phosphoenol pyruvate carboxylase feedback resistant) Phosphoenol Pyruvate Carboxylase feedback resistant)	WO0104325 EP1108790 EP0723011 WO0100852 EP1108790	AX122198 AX127149 AX127148 AX123554

Pict Phosphoglycerate Kinase EC 2.7.2.3 (phosphoglycerate kinase) Protein Kinase A (protein kinase A) Protein Kinase B pknD Protein Kinase B ppl108790 EP1108790 EP1108790 EP1108790 EP1108790 EP1108790 EP1108790 EP1108790 pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase EP1108790 EP10870 EP1108790 EP1108790 E	
(phosphoglycerate kinase) (phosphoglycerate kinase) (phosphoglycerate kinase) (phosphoglycerate kinase) (phosphoglycerate kinase) (phosphoferate kinase A (protein Kinase A (protein kinase A) (protein kinase B (protein kinase B) (protein kinase B) (protein kinase B) (protein kinase B) (protein kinase G) (protein kinase B) EP1108790 EC 2.7.3.9 (phosphoenol pyruvate synthase) EP1108790 EC 2.7.3.9 (phosphotransferase system Enzyme I EP1108790	AX121838
Eikmanns, Journal of Bacteriology 174:6076-6086 (1992) pknB Protein Kinase A (protein kinase A) (protein kinase B) pknD Protein Kinase B (protein kinase B) pknD Protein Kinase B (protein kinase B) pknB Protein Kinase B (protein kinase B) pknB Protein Kinase B (protein kinase B) pknB Protein Kinase G (protein kinase G) pknG Protein Kinase G (protein kinase G) ppsA Phosphoenol Pyruvate Synthase EC 2.7.9.2 (phosphoenol pyruvate synthase) ptsH Phosphotransferase System Protein H EC 2.7.1.69 (phosphotransferase system component H) ptsI Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I) ptsM Glukose-specific Phosphotransferase System Enzyme II EC 2.7.1.69 (glucose phosphotransferase-system enzyme II) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) amino acid exchange P458S sigC Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C) sigD RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor)	AX127148
Journal of Bacteriology 174:6076-6086 (1992) pknA Protein Kinase A (protein kinase A) pknB Protein Kinase B (protein kinase B) pknD Protein Kinase B (protein kinase D) pknG Protein Kinase D (protein kinase C) pknG Protein kinase G (protein kinase G) ppsA Phosphoenol Pyruvate Synthase EP1108790 ptsH Phosphotransferase System Protein H EP1108790 ptsH Phosphotransferase System W00100844 component H) ptsI Phosphotransferase System Enzyme I EC 2.7.1.69 (phosphotransferase System Enzyme I EC 2.7.1.69 (glucose phosphotransferase system enzyme II) ptsM Glukose-specific Phosphotransferase System enzyme II) pty (glucose phosphotransferase system enzyme II) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase EC 6.7.7.6 (extracytoplasmic function alternative sigma factor C) EP1108790	AX064943
pknA Protein Kinase A (1992) pknB Protein kinase A) pknB Protein kinase B (protein kinase B) pknD Protein Kinase G (protein kinase G) pknB Phosphoenol pyruvate synthase EC 2.7.9.2 (phosphoenol pyruvate synthase) ptsH Phosphotransferase System Protein H EC 2.7.1.69 (phosphotransferase system component H) ptsI Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I) ptsM Glukose-specific Phosphotransferase System Enzyme II (pyruvate Carboxylase EC 6.4.1.1 (pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) amino acid exchange P458S sigC Sigma Factor C EC EP1108790 EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C) sigD RNA Polymerase Sigma Factor D EP1108790	X59403
pknA Protein Kinase A (1992) pknB Protein kinase A) pknB Protein kinase B) pknD Protein kinase B) pknD Protein kinase B) pknG Protein kinase B) pknG Protein kinase G (protein kinase G) pknG Phosphoenol Pyruvate Synthase EP1108790 ptsH Phosphotransferase System Protein H EP2108790 ptsH Phosphotransferase System Protein H EP2108790 EC 2.7.1.69 (phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase System Enzyme I) ptsH Phosphotransferase System Enzyme I EP2108790 gc 2.7.3.9 (phosphotransferase System Enzyme I) ptsM Glukose-specific Phosphotransferase System Enzyme II EC 2.7.1.69 (glucose phosphotransferase-system enzyme II) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) amino acid exchange P458S sigC Sigma Factor C EP1108790 EP1108790 EP1108790 EP1108790 EP1108790 EP1108790 EP1108790 EP1108790	
pknA Protein Kinase A (protein kinase A) EP1108790 pknB Protein Kinase B (protein kinase B) pknD Protein Kinase B (protein kinase B) pknG Protein Kinase C (protein kinase G) pknG Phosphoenol Pyruvate Synthase EC 2.7.9.2 (phosphoenol pyruvate synthase) ptsH Phosphotransferase System Protein H EP1108790 EC 2.7.1.69 (phosphotransferase system component H) ptsI Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I) ptsM Glukose-specific Phosphotransferase System Enzyme I EC 2.7.1.69 (glucose phosphotransferase-system enzyme II) ptsM Glukose-specific Phosphotransferase System Enzyme II (1-2):137-145 (1994) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase EP1108790 pyc Pyruvate Carboxylase EP1108790 pyc Pyruvate Carboxylase EP1108790 pyc Pyruvate Carboxylase EP1108790 EP1108790 EP1108790	
pknA Protein Kinase A (protein kinase A) pknB Protein Kinase B (protein kinase B) pknD Protein Kinase D (protein kinase D) pknG Protein Kinase G (protein kinase G) pknB Protein Kinase G (protein kinase G) pknG Protein Kinase G (protein kinase G) ppsA Phosphoenol Pyruvate Synthase EP1108790 EC 2.7.9.2 (phosphoenol pyruvate synthase) ptsH Phosphotransferase System Protein H EP1108790 EC 2.7.1.69 (phosphotransferase system component H) ptsI Phosphotransferase system Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I) ptsM Glukose-specific Phosphotransferase System Enzyme II EC 2.7.1.69 (glucose phosphotransferase-system enzyme II) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C EC 2.7.7.6 (RNA polymerase sigma factor) EigsC Sigma Pactor C EC 2.7.7.6 (RNA polymerase sigma factor)	
pknB Protein Kinase B (protein kinase B) pknD Protein Kinase B) pknD Protein Kinase B (protein kinase B) pknD Protein Kinase D (protein kinase D) pknG Protein Kinase G (protein kinase G) ppsA Phosphoenol Pyruvate Synthase EC 2.7.9.2 (phosphoenol pyruvate synthase) ptsH Phosphotransferase System Protein H EP1108790 EC 2.7.1.69 (phosphotransferase system component H) ptsI Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I) ptsM Glukose-specific Phosphotransferase System Enzyme I EC 2.7.1.69 (glucose phosphotransferase-system enzyme II) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase)	
pknB (protein Kinase B) pknD (protein kinase B) pknD (protein kinase D) (protein kinase D) (protein kinase G) pknG (protein kinase G) ppsA (protein kinase G) ppsA (protein kinase G) ppsA (protein kinase G) ppsA (phosphoenol pyruvate Synthase) ptsH (phosphotransferase System Protein H (phosphotransferase System Protein H (phosphotransferase System Enzyme I) ptsI (phosphotransferase System Enzyme I) ptsI (phosphotransferase System Enzyme I) ptsM (glucose phosphotransferase System enzyme II) ptsM (glucose phosphotransferase System enzyme II) pyc (glucose phosphotransferase-system enzyme II) pyc (pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc (phosphotransferase Sigma factor C) pyc (phosphotransferase Sigma factor D) pyc (phosphotransferase Sigma factor D) pyc (phosphotransferase Sigma factor) pyc (phosphotransferase System Protein H EP1108790 pyc (phosphotransferase System Protein H	AX120131
pknD Protein Kinase B) pknD Protein Kinase D (protein kinase D) pknG Protein Kinase G (protein kinase G) ppsA Phosphoenol Pyruvate Synthase EC 2.7.9.2 (phosphoenol pyruvate synthase) ptsH Phosphotransferase System Protein H EP1108790 (phosphotransferase system component H) ptsI Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I) ptsM Glukose-specific Phosphotransferase System enzyme II ptsM Glukose-specific Phosphotransferase System enzyme II ptsM (glucose phosphotransferase-system enzyme II) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate Carboxylase) pyc Pyruvate Carboxylase pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase pyc Pyruvate Carboxylase EC 2.7.7.6 (extracytoplasmic function alternative sigma factor D EC 2.7.7.6 (RNA polymerase sigma factor)	AX120085
pknD Protein Kinase D (protein kinase D) pknG Protein Kinase G (protein kinase G) ppsA Phosphoenol Pyruvate Synthase EC 2.7.9.2 (phosphoenol pyruvate synthase) ptsH Phosphotransferase System Protein H EP1108790 EC 2.7.1.69 (phosphotransferase system component H) ptsI Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I) ptsM Glukose-specific Phosphotransferase System Enzyme II EC 2.7.1.69 (glucose phosphotransferase-system enzyme II) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) sigC Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor) sigD RNA Polymerase sigma factor) sigC Sigma Factor C EC 2.7.7.6 (RNA polymerase sigma factor)	AX120130
pknG Protein Kinase G (protein kinase G (protein kinase G) ppsA Phosphoenol Pyruvate Synthase EC 2.7.9.2 (phosphoenol pyruvate synthase) ptsH Phosphotransferase System Protein H EP1108790 EC 2.7.1.69 (phosphotransferase system component H) ptsI Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I) ptsM Glukose-specific Phosphotransferase System Enzyme II ptsM Glukose-specific Phosphotransferase System Enzyme II) ptsM Glukose-specific Phosphotransferase System Enzyme II) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate Carboxylase) amino acid exchange P458S sigC Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor D EC 2.7.7.6 (RNA polymerase sigma factor) sigD RNA Polymerase sigma factor)	AX120085
pknG Protein Kinase G (protein kinase G) ppsA Phosphoenol Pyruvate Synthase EC 2.7.9.2 (phosphoenol pyruvate synthase) ptsH Phosphotransferase System Protein H EP1108790 EC 2.7.1.69 (phosphotransferase system component H) ptsI Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase System enzyme I) ptsM Glukose-specific Phosphotransferase System enzyme II) ptsM (glucose phosphotransferase System enzyme II) ptc 2.7.1.69 (glucose phosphotransferase System enzyme II) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase pyc Pyruvate Carboxylase pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase pyc Pyruvate Carboxylase EP1108790	AX127150
Special Region Process	AX122469
Special Region Process	AX122468
Phosphoenol Pyruvate Synthase EC 2.7.9.2 (phosphoenol pyruvate synthase) PtsH Phosphotransferase System Protein H EC 2.7.1.69 (phosphotransferase system component H) PtsI Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I) ptsM Glukose-specific Phosphotransferase System Enzyme II EC 2.7.1.69 (glucose phosphotransferase-system enzyme II) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) PtsH Pyruvate Carboxylase EC 6.4.1.1 (pyruvate Carboxylase) EP1108790 EP1108790 EP1108790 EP1108790 EP1108790 EP1108790 EP1108790 EP1108790 EP1108790 EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C) EC 2.7.7.6 (RNA polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor)	AX127152
EC 2.7.9.2 (phosphotransferase System Protein H EC 2.7.1.69 (phosphotransferase system Component H) ptsI Phosphotransferase System Enzyme I EP1108790 EC 2.7.3.9 (phosphotransferase System Enzyme I EP1108790 EC 2.7.3.9 (phosphotransferase system Enzyme I) ptsM Glukose-specific Phosphotransferase System Enzyme II EC 2.7.1.69 (glucose phosphotransferase-system Enzyme II) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) P458S EC 6.4.1.1 (pyruvate Carboxylase) EP1108790 EP1108790 EP1108790 EC 2.7.7.6 (extracytoplasmic function alternative sigma factor D EC 2.7.7.6 (RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor)	AX123109
Phosphoenol pyruvate synthase Phosphotransferase System Protein H EC 2.7.1.69 (phosphotransferase system component H) PtsI	AX127144
Phosphotransferase System Protein H EP1108790 EC 2.7.1.69 (phosphotransferase system component H) PtsI Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I) ptsM Glukose-specific Phosphotransferase System Enzyme II EC 2.7.1.69 (glucose phosphotransferase-system enzyme II) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) amino acid exchange P458S sigC Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C) SigD RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor)	AX120700
EC 2.7.1.69 (phosphotransferase system component H) ptsI Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase System Enzyme I EC 2.7.1.69 (phosphotransferase system enzyme I) ptsM Glukose-specific Phosphotransferase System Enzyme II	AX122469
(phosphotransferase system component H) ptsI Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I) ptsM Glukose-specific Phosphotransferase System Enzyme II ptsM Glukose-specific Phosphotransferase System Enzyme II EC 2.7.1.69 (glucose phosphotransferase-system enzyme II) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase) pyc Pyruvate Carboxylase pyc Pyruvate Carboxylase pyc Pyruvate Carboxylase pyc Pyruvate Carboxylase pyc Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor D EC 2.7.7.6 (RNA polymerase Sigma factor) sigE Sigma Factor C EC 2.7.7.6 (RNA polymerase sigma factor)	AX122210
component H) ptsI Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I) ptsM Glukose-specific Phosphotransferase System Enzyme II EC 2.7.1.69 (glucose phosphotransferase-system enzyme II) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate Carboxylase) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate Carboxylase) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase pyc Pyruvate Carboxyla	AX127149
PtsI Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I) ptsM Glukose-specific Phosphotransferase System Enzyme II EC 2.7.1.69 (glucose phosphotransferase-system enzyme II) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase EP1108790 pyc Pyruvate Carboxylase EP1108790 EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C) sigD RNA Polymerase Sigma Factor D EP1108790 EC 2.7.7.6 (RNA polymerase sigma factor)	AX069154
EC 2.7.3.9 (phosphotransferase system enzyme I) ptsM Glukose-specific Phosphotransferase System Enzyme II EC 2.7.1.69 (glucose phosphotransferase-system enzyme II) Letters 119 (1-2):137-145 (1994) Letters 119 (1-2):137-145 (1994)	AX122206
enzyme I) ptsM Glukose-specific Phosphotransferase	AX122206 AX127149
enzyme I) ptsM Glukose-specific Phosphotransferase	HA12/149
System Enzyme II EC 2.7.1.69 (glucose phosphotransferase-system enzyme II) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) amino acid exchange P458S sigC Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C) sigD RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor) sigE Sigma Factor C EC 2.7.7.6 (RNA polymerase sigma factor)	
System Enzyme II EC 2.7.1.69 (glucose phosphotransferase-system enzyme II) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) amino acid exchange P458S sigC Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C) sigD RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor) sigF Sigma Factor B EP1108790	S L18874
EC 2.7.1.69 (glucose phosphotransferase-system enzyme II) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) amino acid exchange P458S sigC Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C) sigD RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor) sigE Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor)	
enzyme II) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) pyc Pyruvate Carboxylase) pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate Carboxylase) EC 6.4.1.1 (pyruvate carboxylase) amino acid exchange P458S sigC Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C) sigD RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor) sigE Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor)	
pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) Pyc Pyruvate Carboxylase Pyc Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) EC 6.4.1.1 (pyruvate carboxylase) amino acid exchange P458S SigC Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C) SigD RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor) EP1108790 EP1108790	
EC 6.4.1.1 (pyruvate carboxylase) Peters-Wendisch et al., Microbiology 144:915-927 (1998) Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) amino acid exchange P458S SigC Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C) SigD RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor) SigE Sigma Factor D EXAMPLE S	
(pyruvate carboxylase) pyc Pyruvate Carboxylase EP1108790 pyc Pyruvate carboxylase EP1108790 EC 6.4.1.1 (pyruvate carboxylase) amino acid exchange P458S sigC Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C) sigD RNA Polymerase Sigma Factor D EP1108790 EC 2.7.7.6 (RNA polymerase sigma factor) SigE Sigma Factor Reserve Reserve Reserved Factor D EP1108790	A97276
pyc Pyruvate Carboxylase EP1108790 EC 6.4.1.1 (pyruvate carboxylase) amino acid exchange P458S sigC Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C) sigD RNA Polymerase Sigma Factor D EP1108790 EC 2.7.7.6 (RNA polymerase sigma factor)	Y09548
pyc Pyruvate Carboxylase P458S EC 6.4.1.1 (pyruvate carboxylase) amino acid exchange P458S sigC Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C) sigD RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor)	
pyc Pyruvate Carboxylase EP1108790 P458S EC 6.4.1.1 (pyruvate carboxylase) amino acid exchange P458S sigC Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C) sigD RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor) SigF Sigma Factor R	
Pyruvate Carboxylase P458S EC 6.4.1.1 (pyruvate carboxylase) amino acid exchange P458S sigC Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C) sigD RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor)	
P458S EC 6.4.1.1 (pyruvate carboxylase) amino acid exchange P458S sigC Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C) sigD RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor)	
(pyruvate carboxylase) amino acid exchange P458S sigC Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C) sigD RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor)	
amino acid exchange P458S sigC Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C) sigD RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor)	
sigC Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C) sigD RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor) sigE Sigma Factor R	
EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C) sigD RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor)	
(extracytoplasmic function alternative sigma factor C) sigD RNA Polymerase Sigma Factor D EP1108790 EC 2.7.7.6 (RNA polymerase sigma factor)	AX120368
alternative sigma factor C) sigD RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor) SigE Sigma Factor P	AX120085
sigD RNA Polymerase Sigma Factor D EP1108790 EC 2.7.7.6 (RNA polymerase sigma factor)	
EC 2.7.7.6 (RNA polymerase sigma factor)	27100000
(RNA polymerase sigma factor)	AX120753
sign Ciama Packer B	AX127144
sigE Sigma Factor E EP1108790	77127146
EC 2.7.7.6	AX127146 AX121325
(extracytoplasmic function	MAT41342
alternative sigma factor E)	
sigH Sigma Factor H EP1108790	AX127145
EC 2.7.7.6	AX12/145 AX120939
(sigma factor SigH)	, , , , , , , , , , , , , , , , , , ,
sigM Sigma Factor M	AX123500

WO 03/040373 PCT/EP02/08464

(sigma factor SigM) tal Transaldolase EC 2.2.1.2 (transaldolase) thrB Homoserine Kinase EC 2.7.1.39 (homoserine kinase) thrC Threonine Synthase EC 4.2.99.2 (threonine synthase) (threonine synthase) EC 4.2.99.2 (threonine synthase) MO0104325 Peoples et al., Molecular Microbiology 2:63-72 (1988) Han et al., Molecular Microbiology 4:1693-1702 (1990)		EC 2.7.7.6		AX127153
tal Transaldolase EC 2.2.1.2 (transaldolase) thrB Homoserine Kinase EC 2.7.1.39 (homoserine kinase) thrC Threonine Synthase EC 4.2.99.2 (threonine synthase)	(sigma factor SigM)			
thrB Homoserine Kinase Peoples et al., Molecular Microbiology 2:63-72 (1988) thrC Threonine Synthase EC 4.2.99.2 Molecular Microbiology 4:1693-1702 (1990)	tal		WO0104325	AX076272
thrB Homoserine Kinase Peoples et al., Molecular Microbiology 2:63-72 (1988) thrC Threonine Synthase EC 4.2.99.2 Molecular Microbiology 4:1693-1702 (1990)		EC 2.2.1.2		
thrB Homoserine Kinase EC 2.7.1.39 (homoserine kinase) Threonine Synthase EC 4.2.99.2 (threonine synthase) Microbiology 2:63-72 (1988) Han et al., Molecular Microbiology 4:1693-1702 (1990)		(transaldolase)		
EC 2.7.1.39 (homoserine kinase) Threonine Synthase EC 4.2.99.2 (threonine synthase) Molecular 2:63-72 (1988) Han et al., Molecular Microbiology 4:1693-1702 (1990)	thrB		Peoples et al.,	Y00546
thrC Threonine Synthase Han et al., X56037 EC 4.2.99.2 Molecular (threonine synthase) Microbiology 4:1693-1702 (1990)		EC 2.7.1.39		
thrC Threonine Synthase Han et al., X56037 EC 4.2.99.2 Molecular (threonine synthase) Microbiology 4:1693-1702 (1990)		(homoserine kinase)	Microbiology	
EC 4.2.99.2 Molecular (threonine synthase) Microbiology 4:1693-1702 (1990)			2:63-72 (1988)	
(threonine synthase) Microbiology 4:1693-1702 (1990)	thrC	Threonine Synthase	Han et al.,	X56037
4:1693-1702 (1990)		EC 4.2.99.2	Molecular	
(1990)		(threonine synthase)	Microbiology	
			4:1693-1702	
			(1990)	<u> </u>
thrE Threonine Exporter EP1085091 AX137526	thrE	Threonine Exporter	EP1085091	AX137526
(threonine export carrier)		(threonine export carrier)		
	thyA	Thymidylate Synthase	EP1108790	AX121026
EC 2.1.1.45 AX127145		EC 2.1.1.45		AX127145
(thymidylate synthase)				
	tkt	Transketolase		AB023377
EC 2.2.1.1 NCBI		•	NCBI	
(transketolase)				
tpi Triose phosphate Isomerase Eikmanns, X59403	tpi		1	X59403
EC 5.3.1.1 Journal of				
(triose phosphate isomerase) Bacteriology		(triose phosphate isomerase)		
174:6076-6086				
(1992)	4	0-11 0-12 7-14-1		hv122701
	zwal		ELITITOS	AX133781
zwf Glucose 6-Phosphate 1-Dehydrogenase EP1108790 AX127148			ED1109700	AX127148
	ZWI	i de la companya de	FLTTAQ120	AX12/148 AX121827
			 WO0104325	AX076272
(glucose 6-phosphate 1- WO0104325 AX076272 dehydrogenase)			MOOTARRA	AAUIUZIZ
zwf Glucose 6-Phosphate 1-Dehydrogenase EP1108790			EP1108790	
A213T EC 1.1.1.49		-	DETTOOLSO	
(glucose 6-phosphate 1-	ひかてつて			
dehydrogenase)			Ī	Ī
amino acid exchange A213T	, _ ,	_ _		

Table 7

Target sites for integration of open reading frames, genes and alleles of threonine production

Gene	Description of the coded	Reference	Access
name	enzyme or protein	Kerer ence	Number
2202110	citagine of process		Number
ccpA1	Catabolite Control	WO0100844	AX065267
CCDAI	Protein	EP1108790	AX127147
	(catabolite control	EFITOOISU	HVT7/T4/
	protein A1)		
ccpA2	Catabolite Control	WO0100944	24065067
CCDAZ	Protein	WO0100844 EP1108790	AX065267
	(catabolite control	EP1108/90	AX121594
	•		
citA	protein A2) Sensor Kinase CitA	7771100700	777 001 61
CILA		EP1108790	AX120161
citB	(sensor kinase CitA)	TD1100700	NY120162
CILE	Transcription Regulator CitB	EP1108790	AX120163
	, — — — .		
	(transcription regulator		
citE	CitB)	T-1001 000 4 A	77065401
CILE	Citrate Lyase	WO0100844	AX065421
	EC 4.1.3.6	EP1108790	AX127146
ddh	(citrate lyase)	Tabina ab al Arealaia	007704
udii	Diaminopimelate Dehydrogenase	Ishino et al., Nucleic Acids Research 15: 3917	S07384
	EC 1.4.1.16	1	AX12/132
	(diaminopimelate	. (1987) EP1108790	
	dehydrogenase)	EP1108/90	
gluA	Glutamate Transport ATP-	Vronomovor of al	X81191
grun	binding Protein	Kronemeyer et al., Journal of Bacteriology	VOTTAT
	(glutamate transport ATP-	177(5):1152-8 (1995)	
	binding protein)	177(3):1132-0 (1333)	
gluB	Glutamate-binding Protein	Kronemeyer et al.,	x81191
9.40	(glutamate-binding	Journal of Bacteriology	AUTIJI
	protein)	177(5):1152-8 (1995)	/
gluC	Glutamate Transport	Kronemeyer et al.,	X81191
grac	Permease	Journal of Bacteriology	AOILJI
	(glutamate transport	177(5):1152-8 (1995)	
	system permease)	177(37.1132 6 (1333)	
gluD	Glutamate Transport	Kronemeyer et al.,	X81191
, 91W	Permease	Journal of Bacteriology	2501131
	(glutamate transport	177(5):1152-8 (1995)	
	system permease)		
glyA	Glycine Glycine	WO0100843	AX063861
9~3**	Hydroxymethyltransferase	MOOTOOGO	AF327063
	EC 2.1.2.1		12.02.1005
1	(glycine		
	hydroxymethyltransferase)		
ilvA	Threonine Dehydratase	Möckel et al., Journal	A47044
	EC 4.2.1.16	of Bacteriology 174	L01508
	(threonine dehydratase)	(24), 8065-8072 (1992)	AX127150
	(commic definationse)	EP1108790	
ilvBN	Acetolactate Synthase	Keilhauer et al.,	L09232
1 TO A 1711	EC 4.1.3.18	Journal of Bacteriology	
L	1 ~~ 4 . 4 . 4 . 4 . 4	COUTTAIN OF DUCCETTOTORY	

	/	14DE (4D) EEDE (4000)	
	(acetolactate synthase)	175(17):5595-603 (1993)	NY107147
ilvC	Reductoisomerase	EP1108790 Keilhauer et al.,	AX127147 C48648
4140	EC 1.1.1.86	Journal of Bacteriology	
	(ketol-acid	175 (17):5595-603 (1993)	AXIZILEI
	reductoisomerase)	EP1108790	
ilvD	Dihydroxy-acid	EP1006189	AX136925
	Dehydratase	EP1000183	AX130323
	EC 4.2.1.9		
	(dihydroxy-acid		
	dehydratase)	•	
luxR	Transcription Regulator	WO0100842	AX065953
- W.	LuxR	EP1108790	AX123320
	(transcription regulator	EFILOUIDO	AKIZJJZU
	LuxR)		
luxS	Histidine Kinase LuxS	EP1108790	AX123323
~ W1D	(histidine kinase LuxS)	BF 1100/30	AX127153
	(Milboratic Milabe Land)		AMIZ/IJJ
lysR1	Transcription Regulator	EP1108790	AX064673
	LysR1	111100,30	AX127144
	(transcription regulator		12122/144
	LysR1)		
lysR2	Transcription Activator	EP1108790	AX123312
- 3	LysR2		
	(transcription regulator		
	LysR2)		
lysR3	Transcription Regulator	WO0100842	AX065957
	LysR3	EP1108790	AX127150
	(transcription regulator		
	LysR3)		
mdh	Malate Dehydrogenase	WO0100844	AX064895
	EC 1.1.1.37		
•	(malate dehydrogenase)	1	
menE	O-Succinylbenzoic Acid	WO0100843	AX064599
	CoA Ligase	EP1108790	AX064193
	EC 6.2.1.26		AX127144
	(O-succinylbenzoate CoA		
	ligase)		
metA	Homoserine O-	Park et al., Molecular	AX063895
	Acetyltransferase	Cells 30;8(3):286-94	AX127145
	EC 2.3.1.31	(1998)	
	(homoserine O-	WO0100843	
	acetyltransferase)	EP1108790	
metD	Transcription Regulator	EP1108790	AX123327
	MetD		AX127153
	(transcription regulator		
	MetD)		
pck	Phosphoenol Pyruvate	WO0100844	AJ269506
	Carboxykinase		AX065053
	(phosphoenol pyruvate		
	carboxykinase)		
рохВ	Pyruvate Oxidase	WO0100844	AX064959
	EC 1.2.3.3	EP1096013	AX137665
	(pyruvate oxidase)		
· · · · · · · · · · · · · · · · · · ·		• · · · · · · · · · · · · · · · · · · ·	3 3 5 4 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6
sigB	RNA Polymerase	EP1108790	AX127149
sigB	RNA Polymerase Transcription Factor	EP1108790	AX12/149
sigB	-	EP1108790	AX12/149

zwa2	Cell Growth Factor 2	EP1106693	AX113822
	(growth factor 2)	EP1108790	AX127146

The invention accordingly also provides a process for the production of coryneform bacteria which produce L-methionine and/or L-threonine, which comprises

- isolating the nucleotide sequence of at least one desired ORF, gene or allele of methionine production or threonine production, optionally including the expression and/or regulation signals,
- b) providing the 5' and the 3' end of the ORF, gene or allele with nucleotide sequences of the target site,
 - c) preferably incorporating the nucleotide sequence of the desired ORF, gene or allele provided with nucleotide sequences of the target site into a vector which does not replicate or replicates to only a limited extent in coryneform bacteria,
 - d) transferring the nucleotide sequence according to b) or c) into coryneform bacteria, and

15

30

e) isolating coryneform bacteria in which the nucleotide sequence according to a) is incorporated at the target site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the target site.

The invention furthermore provides coryneform bacteria, in particular of the genus Corynebacterium, which produce L-valine, wherein these have, in addition to at least one of the copy of an open reading frame (ORF), gene or allele of valine production present at the natural site (locus), in each case a second, optionally third or fourth copy of the

open reading frame (ORF), gene or allele in question at in each case a second, optionally third or fourth site in integrated form, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular second, optionally third or fourth site.

The invention also furthermore provides a process for the preparation of L-valine, which comprises the following steps:

fermentation of coryneform bacteria, in particular a) Corynebacterium glutamicum, characterized in that these have, in addition to at least one of the copy of 15 an open reading frame (ORF), gene or allele of valine production present at the natural site (locus), in each case a second, optionally third or fourth copy of the open reading frame (ORF), gene or allele in question at in each case a second, optionally third or 20 fourth site in integrated form, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being 25 present at the particular second, optionally third or fourth site,

under conditions which allow expression of the said open reading frames (ORF), genes or alleles,

- 30 b) concentration of the L-valine in the fermentation broth,
 - c) isolation of the L-valine from the fermentation broth, optionally

d) with constituents from the fermentation broth and/or the biomass to the extent of > (greater than) 0 to 100%.

A "copy of an open reading frame (ORF), gene or allele of valine production" is to be understood as meaning all the open reading frames, genes or alleles of which enhancement/over-expression can have the effect of improving valine production.

These include, inter alia, the following open reading

frames, genes or alleles: brnE, brnF, brnEF, cstA, cysD,
dps, eno, fda, gap, gap2, gdh, ilvB, ilvN, ilvBN, ilvC,
ilvD, ilvE msiK, pgk, ptsH, ptsI, ptsM, sigC, sigD, sigE,
sigH, sigM, tpi, zwal. These are summarized and explained
in Table 8. These include in particular the acetolactate
synthase which codes for a valine-resistant.

The second, optionally third or fourth copy of the open reading frame (ORF), gene or allele of threonine production in question can be integrated at in each case a second, optionally third or fourth site. The following open reading frames, genes or nucleotide sequences, inter alia, can be used for this: aecD, ccpA1, ccpA2, citA, citB, citE, ddh, gluA, gluB, gluC, gluD, glyA, ilvA, luxR, lysR1, lysR2, lysR3, panB, panC, poxB and zwa2. These are summarized and explained in Table 9.

20

The sites mentioned include, of course, not only the coding regions of the open reading frames or genes mentioned, but also the regions or nucleotide sequences lying upstream which are responsible for expression and regulation, such as, for example, ribosome binding sites, promoters, binding sites for regulatory ribonucleic acids and attenuators. These regions in general lie in a range of 1-800, 1-600, 1-400, 1-200, 1-100 or 1-50 nucleotides upstream of the coding region. In the same way, regions lying downstream, such as, for example,

transcription terminators, are also included. These regions in general lie in a range of 1-400, 1-200, 1-100, 1-50 or 1-25 nucleotides downstream of the coding region.

Intergenic regions in the chromosome, that is to say nucleotide sequences without a coding function, can furthermore be used. Finally, prophages or defective phages contained in the chromosome can be used for this.

Table 8
Open reading frames, genes and alleles of valine production

Name	Description of the coded enzyme or	Reference	Access
	protein		Number
			Number
brnEF	Export of branched-chain amino	EP1096010	
1	acids		
]	(branched chain amino acid export)	Kennerknecht et	AF454053
		al., NCBI	W 424022
cstA	Carbon Starvation Protein A	EP1108790	AX120811
}	(carbon starvation protein A)	WO0100804	AX066109
dps	DNA Protection Protein	EP1108790	AX127153
	(protection during starvation	E 1100/30	BYTS 1772
	protein)		
eno	Enolase	EP1108790	AX127146
	EC 4.2.1.11	WO0100844	AX064945
	(enolase)	EP1090998	AX136862
	(Chorase)	Hermann et al.,	AAT30002
		Electrophoresis	
		19:3217-3221	
		(1998)	<u> </u>
fda	Fructose Bisphosphate Aldolase	van der Osten et	X17313
	EC 4.1.2.13	al., Molecular	1117313
	(fructose bisphosphate aldolase)	Microbiology	}
	}	3:1625-1637	
		(1989)	
gap	Glyceraldehyde 3-Phosphate	EP1108790	AX127148
	Dehydrogenase	WO0100844	AX064941
	EC 1.2.1.12	Eikmanns et al.,	X59403
	(glyceraldehyde 3-phosphate	Journal of	
	dehydrogenase)	Bacteriology	
		174:6076-	1
		6086 (1992)	
gap2	Glyceraldehyde 3-Phosphate	EP1108790	AX127146
	Dehydrogenase	WO0100844	AX064939
	EC 1.2.1.12		
	(glyceraldehyde 3-phosphate		
	dehydrogenase 2)		
gdh	Glutamate Dehydrogenase	EP1108790	AX127150
	EC 1.4.1.4	WO0100844	AX063811
	(glutamate dehydrogenase)	Boermann et al.,	X59404
		Molecular	
		Microbiology	
		6:317-326	
		(1992);	
		Guyonvarch et	X72855
		al., NCBI	
ilvBN	Acetolactate Synthase	Keilhauer et	L09232
	EC 4.1.3.18	al., Journal of	
	(acetolactate synthase)	Bacteriology	
		175(17):5595-603	
		(1993)	
		EP1108790	AX127147
ilvC	Isomeroreductase	Keilhauer et	C48648
ſ	EC 1.1.1.86	al., Journal of	AX127147

	(acetohydroxy acid	Dochowielens	
	isomeroreductase)	Bacteriology	
	isomeroreductase)	175(17):5595-603	
		(1993)	1
		EP1108790	
ilvD	Dihydroxy-acid Dehydratase	EP1006189	AX136925
	EC 4.2.1.9		}
	(dihydroxy acid dehydratase)		
ilvE	Transaminase B	EP1108790	AX127150
	EC 2.6.1.42		AX122498
	(transaminase B)		
msiK	Sugar Importer	EP1108790	AX120892
	(multiple sugar import protein)]	
pgk	Phosphoglycerate Kinase	EP1108790	AX121838
pgr	EC 2.7.2.3	ELTIOO120	AX127148
		1300100844	
	(phosphoglycerate kinase)	WO0100844	AX064943
		Eikmanns,	X59403
		Journal of	
		Bacteriology	·
		174:6076-6086	
		(1992)	
ptsH	Phosphotransferase System Protein H	EP1108790	AX122210
	EC 2.7.1.69		AX127149
	(phosphotransferase system	WO0100844	AX069154
	component H)		
ptsI	Phosphotransferase System Enzyme I	EP1108790	AX122206
PCDT	EC 2.7.3.9		AX127149
	(phosphotransferase system		IMILE / LEJ
	enzyme I)		
	<u> </u>	I on at all DEMC	L18874
ptsM	Glucose-specific Phosphotransferase	Lee et al., FEMS	DT0014
	System Enzyme II	Microbiology	
	EC 2.7.1.69	Letters 119	
	(glucose phosphotransferase-system	(1-2):137-145	
	enzyme II)	(1994)	
sigC	Sigma Factor C	EP1108790	AX120368
	EC 2.7.7.6		AX120085
	(extracytoplasmic function		
	alternative sigma factor C)		
sigD	RNA Polymerase Sigma Factor D	EP1108790	AX120753
	EC 2.7.7.6		AX127144
	(RNA polymerase sigma factor)		
sigE	Sigma Factor E	EP1108790	AX127146
	EC 2.7.7.6		AX121325
	(extracytoplasmic function		
	alternative sigma factor E)		
oi all		EP1108790	AX127145
sigH	Sigma Factor H	 PLTTAG120	
	EC 2.7.7.6	}	AX120939
	(sigma factor SigH)		
sigM	Sigma Factor M	EP1108790	AX123500
	EC 2.7.7.6		AX127153
	(sigma factor SigM)		
tpi	Triose Phosphate Isomerase	Eikmanns,	X59403
	EC 5.3.1.1	Journal of	
	(triose phosphate isomerase)	Bacteriology	į
		174:6076-6086	
		(1992)	
zwa1	Cell Growth Factor 1	EP1111062	AX133781
CWOT		EFITIEUZ	LECTABLET
	(growth factor 1)	1	

WO 03/040373 PCT/EP02/08464

Table 9

Target sites for integration of open reading frames, genes and alleles of valine production

Deta C-S Lyase Rossol et al., Journal M8 EC 2.6.1.1 of Bacteriology 174(9):2968-77 (1992) Rossol et al., Journal M8 Of Bacteriology 174(9):2968-77 (1992) Rossol et al., Journal M8 Of Bacteriology Rossol et al., Journal M8 Rossol et al., Journal Rossol et al., Journal M8 Rossol et al., Journal Rossol et	Access	Reference	Description of the coded	Gene
CC 2.6.1.1 Of Bacteriology (beta C-S lyase) 174(9):2968-77 (1992)	Number		enzyme or protein	name
CC 2.6.1.1 Of Bacteriology (beta C-S lyase) 174(9):2968-77 (1992)		1		
CCPA1 Catabolite Control W00100844 AX0 EP1108790 AX1 Catabolite Control Protein AX1 EP1108790 AX1 CitB CitTate Lyase W00100844 AX0 EP1108790 AX1 CitB CitTate Lyase CitB CitTate Lyase CitB CitTate Lyase CitB CitTate Lyase CitB Cit	M89931	Rossol et al., Journal	beta C-S Lyase	aecD
ccpA1 Catabolite Control Protein (catabolite control protein AI) ccpA2 Catabolite Control Protein (catabolite control protein AI) ccpA2 Catabolite Control Protein (catabolite control protein A2) citA Sensor Kinase CitA (sensor kinase CitA) citB Transcription Regulator CitB (transcription regulator CitB (transcription regulator CitB) citE Citrate Lyase W00100844 AX0 (citrate lyase) ddh Diaminopimelate EC 1.4.1.16 (diaminopimelate Dehydrogenase EC 1.4.1.16 (diaminopimelate Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding protein) gluB Glutamate Transport ATP-binding protein (glutamate transport ATP-binding Protein (glutamate Transport ATP-binding Protein) gluC Glutamate Transport ATP-binding Protein (glutamate Transport ATP-binding Protein) gluC Glutamate Transport ATP-binding Protein (glutamate Transport ATP-binding Protein) gluC Glutamate Transport ATP-binding Protein (glutamate Transport System permease) gluD Glutamate Transport Kronemeyer et al., X8 (glutamate Transport System permease) gluD Glutamate Transport Kronemeyer et al., Journal of Bacteriology (glutamate Transport System permease) gluD Glutamate Transport Kronemeyer et al., Journal of Bacteriology (glutamate Transport System permease) gluA Glycine W00100843 AX0 AF3 AF3		of Bacteriology	<u> </u>	
ccpAl Catabolite Control Protein (catabolite control protein Al) ccpA2 Catabolite Control Protein (catabolite control protein Al) ccpA2 Catabolite Control EP1108790 AX1 (catabolite control protein A2) citA Sensor Kinase CitA (sensor kinase CitA) citB Transcription Regulator CitB (transcription regulator CitB (transcription regulator CitB) citE Citrate Lyase W00100844 AX0 (citrate lyase) ddh Diaminopimelate EC 1.4.1.6 (diaminopimelate Dehydrogenase EC 1.4.1.16 (diaminopimelate dehydrogenase) gluA Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding Protein) gluB Glutamate Transport ATP-binding protein) gluC Glutamate Transport ATP-binding protein) gluC Glutamate Transport ATP-protein (glutamate transport ATP-protein) gluC Glutamate Transport ATP-protein (glutamate Transport ATP-protein (glutamate Transport ATP-protein ATP-protein) gluC Glutamate Transport ATP-protein (glutamate Transport ATP-protein ATP-protei		174(9):2968-77 (1992)	(beta C-S lyase)	
(catabolite control protein A1) ccpA2 Catabolite Control Protein (catabolite control protein A2) citA Sensor Kinase CitA (sensor kinase CitA) citB Transcription Regulator CitB (transcription regulator CitB) citE Citrate Lyase EC 4.1.3.6 (citrate lyase) ddh Diaminopimelate Dehydrogenase Acids Research 15: 3917 (1987) citE Cit.4.1.16 (diaminopimelate Bunding Protein (glutamate Transport ATP-binding Protein (glutamate-binding Protein (glutamate Transport ATP-binding protein) gluB Glutamate Transport ATP-binding protein) gluC Glutamate Transport Permease (glutamate transport System permease) gluD Glutamate Transport Permease (glutamate transport System permease) glyA Glycine Hydroxymethyltransferase EC 2.1.2.1	AX065267	WO0100844	Catabolite Control	ccpA1
protein A1) ccpA2 Catabolite Control Protein (catabolite control protein A2) citA Sensor Kinase CitA (sensor kinase CitA) citB Transcription Regulator CitB (transcription regulator CitB) citE Citrate Lyase W00100844 AX0 EP1108790 AX1 (citrate Lyase) citE Citrate Lyase W00100844 AX0 EP1108790 AX1 (citrate lyase) ddh Diaminopimelate Dehydrogenase Acids Research 15: 3917 (1987) EC 1.4.1.16 (diaminopimelate Dehydrogenase) gluA Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding protein) gluB Glutamate Transport ATP-binding protein) gluC Glutamate Transport ATP-binding Protein (glutamate-binding protein) gluC Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding Protein) gluC Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding Protein) gluC Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding Protein) gluC Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding Protein) gluC Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding Protein) gluC Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding Protein) gluC Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding Protein) gluC Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding Protein) gluC Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding Protein) gluC Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding Protein) gluC Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding Protein) gluC Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding Protein) gluC Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding Protein ATP-b	AX127147	EP1108790	Protein	
ccpa2 Catabolite Control Protein (catabolite control protein A2) citA Sensor Kinase CitA (sensor kinase CitA) citB Transcription Regulator CitB (transcription regulator CitB) citC Citrate Lyase EC 4.1.3.6 (citrate lyase) ddh Diaminophmelate Dehydrogenase EC 1.4.1.16 (diaminophmelate dehydrogenase) gluA Glutamate Transport ATP- binding Protein (glutamate-binding protein) gluB Glutamate Transport Pormease (glutamate Transport Pormease (glutamate transport Pormease (glutamate transport System permease) gluA Glutamate transport gluC Glutamate Transport Pormease (glutamate Transport Pormease (glutamate transport System permease) gluD Glutamate transport Pormease (glutamate transport System permease) glyA Glycine WO0109843 AX0 AX1 EP1108790 AX1 EP1108790 AX1 EP1108790 AX1 (1987) AX1 (1987) AX1 (1987) AX2 (1987) AX1 (1987) AX2 (1987) AX2 (1987) AX3 AX4 AX5 AX6 AX6 AX7 AX8		į	(catabolite control	
Protein (catabolite control protein A2) citA Sensor Kinase CitA (sensor kinase CitA) citB Transcription Regulator (transcription regulator (citB) citE Citrate Lyase (citrate lyase) ddh Diaminopimelate (diaminopimelate (diaminopimelate dehydrogenase) gluA Glutamate Transport ATP-binding Protein (glutamate-binding protein) gluB Glutamate Transport ATP-binding protein) gluC Glutamate Transport (glutamate transport (glutam			protein A1)	
(catabolite control protein A2) cith Sensor Kinase Cith (sensor kinase Cith) cith Transcription Regulator Cith (transcription regulator Cith (transcription regulator Cith) cith Citrate Lyase W00100844 Ax0 EC 41.3.6 EP1108790 AX1 cith Citrate Lyase W00100844 Ax0 EC 41.3.6 EP1108790 AX1 cith Citrate Lyase W00100844 Ax0 EC 4.1.3.6 EP1108790 AX1 cith Citrate Lyase W00100844 Ax0 EC 1.4.1.6 (citrate lyase) Ishino et al., Nucleic S0 Acids Research 15: 3917 AX1 (1987) (diaminopimelate Acids Research 15: 3917 AX1 (1987) (diaminopimelate BP1108790 AX1 (glutamate Transport ATP-binding Protein (glutamate transport ATP-binding Protein) (glutamate-binding Protein (glutamate-binding Protein (glutamate-binding Protein) GluC Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding Protein) GluC Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding Protein) GluC Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding Protein) GluC Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding Protein) GluC Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding Protein) GluC Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding Protein) GluC Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding Protein) GluC Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding Protein ATP-binding	AX065267	WO0100844	Catabolite Control	ссрА2
citA Sensor Kinase CitA (sensor kinase CitA) citB Transcription Regulator CitB (transcription regulator CitB (transcription regulator CitB) citE Citrate Lyase W00100844 AX1 EP1108790 AX1 citE Citrate Lyase W00100844 AX1 EP1108790 AX1 EC 4.1.3.6 (citrate lyase) ddh Diaminopimelate Ishino et al., Nucleic Acids Research 15: 3917 AX1 EC 1.4.1.16 (1987) EP1108790 dehydrogenase EC 1.4.1.16 (1987) EP1108790 gluA Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding Protein) gluB Glutamate-binding Protein (glutamate-binding Protein) (glutamate-binding Protein) (glutamate Transport ATP-binding	AX121594	EP1108790	Protein	
CitASensor Kinase CitA (sensor kinase CitA)EP1108790AX1CitBTranscription Regulator (transcription regulator CitB)EP1108790AX1CitECitrate Lyase EC 4.1.3.6 (citrate lyase)W00100844 EP1108790AX0ddhDiaminopimelate Dehydrogenase EC 1.4.1.16 (diaminopimelate dehydrogenase)Ishino et al., Nucleic Acids Research 15: 3917 (1987) EP1108790AX1gluAGlutamate Transport ATP- binding Protein (glutamate transport ATP- binding protein)Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)X8gluBGlutamate-binding protein)Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)X8gluCGlutamate Transport Permease (glutamate transport system permease)Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)X8gluDGlutamate Transport Permease (glutamate transport system permease)Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)X8gluDGlutamate Transport Permease (glutamate transport system permease)Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)X8glyAGlycine Hydroxymethyltransferase EC 2.1.2.1W00100843 AX0AX0		}	(catabolite control	
(sensor kinase CitA) citB Transcription Regulator CitB (transcription regulator CitB) citE Citrate Lyase W00100844 AX0 EP1108790 AX1 (citrate lyase) ddh Diaminopimelate Sec Acids Research 15: 3917 AX1 (1987) (diaminopimelate Acids Research 15: 3917 AX1 (diaminopimelate Acids Res			protein A2)	
citB Transcription Regulator CitB (transcription regulator CitB) citE Citrate Lyase EC 4.1.3.6 (citrate lyase) ddh Diaminopimelate Dehydrogenase EC 1.4.1.16 (diaminopimelate dehydrogenase) gluA Glutamate Transport ATP- binding Protein (glutamate-binding protein) gluB Glutamate Transport Permease (glutamate transport So Acids Research 15: 3917 AX1 (1987) EP1108790 AX1 AX2 AX3 AX4 AX4 AX5 AX6 AX6 EP1108790 AX1 AX6 AX7 AX7 AX8 Journal of Bacteriology 177(5):1152-8 (1995) AX8 Journal of Bacteriology 177(5):1152-8 (1995) AX9 AX9 Glydine Hydroxymethyltransferase EC 2.1.2.1	AX120161	EP1108790	Sensor Kinase CitA	citA
CitB (transcription regulator CitB) CitE Citrate Lyase EC 4.1.3.6 (citrate lyase) ddh Diaminopimelate Dehydrogenase EC 1.4.1.16 (diaminopimelate Dehydrogenase) GluA Glutamate Transport ATP- Dinding Protein (glutamate transport ATP- Dinding protein) Glutamate-binding Protein (glutamate-binding protein) Glutamate Transport EC Glutamate Transport Glutamate Transport Glutamate Transport Fermease (glutamate transport System permease) GlyA Glycine Hydroxymethyltransferase EC 2.1.2.1 WOO100844 AX0 EP1108790 AX1 (1987)		<u></u>	(sensor kinase CitA)	
(transcription regulator CitB) CitE Citrate Lyase EC 4.1.3.6 (citrate lyase) ddh Diaminopimelate Dehydrogenase EC 1.4.1.16 (diaminopimelate dehydrogenase) GluA Glutamate Transport ATP- binding Protein (glutamate transport ATP- binding protein) GluB Glutamate-binding Protein (glutamate-binding protein) GluC Glutamate Transport Permease (glutamate transport System permease) GluC Glutamate Transport Permease (glutamate transport System permease) GluC Glutamate Transport Permease (glutamate Transport Permeas	AX120163	EP1108790	Transcription Regulator	citB
CitB) CitE Citrate Lyase EC 4.1.3.6 (citrate lyase) ddh Diaminopimelate Dehydrogenase EC 1.4.1.16 (diaminopimelate dehydrogenase) GluA Glutamate Transport ATP- binding Protein (glutamate-binding protein) GluC Glutamate Transport Permease (glutamate transport System permease) GlyA Glycine Hydroxymethyltransferase EC 2.1.2.1 Ishino et al., Nucleic Acids Research 15: 3917 AX1 (I987) (EP1108790 AX1 (I987) (Fronemeyer et al., Journal of Bacteriology (IPS) AX1 (IPS) AX1 (IPS) (IPS) AX2 (IPS) (IPS) AX3 (IPS) AX4 (IPS) (IPS) AX4 (IPS) (IPS) AX5 (IPS) AX6 (IPS) AX7 AX9 AX0 AX0 AX0 AX0 AX0 AX0 AX3			CitB	
citE Citrate Lyase EC 4.1.3.6 (citrate lyase) ddh Diaminopimelate Ishino et al., Nucleic SO Dehydrogenase Acids Research 15: 3917 AX1 (1987) EC 1.4.1.16 (1987) (diaminopimelate dehydrogenase) gluA Glutamate Transport ATP-binding Protein (glutamate-binding protein) gluB Glutamate-binding protein (glutamate-binding protein) gluC Glutamate Transport ATP-bermease (glutamate transport system permease) gluD Glutamate Transport ATP-bermease (glutamate transport System permease) gluD Glutamate Transport (glutamate transport System permease) glyA Glycine (Hydroxymethyltransferase) GlyA Glycine (MO0109843 AX0 AF3)			(transcription regulator	
EC 4.1.3.6 (citrate lyase) ddh Diaminopimelate Dehydrogenase EC 1.4.1.16 (diaminopimelate dehydrogenase) gluA Glutamate Transport ATP- binding Protein (glutamate-binding protein) gluB Glutamate-binding protein) gluC Glutamate Transport Permease (glutamate transport Permease (glutamate transport Permease (glutamate transport System permease) gluA Glycine Hydroxymethyltransferase EC 2.1.2.1 EP1108790 AX1 (1987) (1987) EP1108790 AX1 (1987) AX1 AX1 (1987) AX1 AX1 (1987) AX1 AX1 AX1 AX1 AX2 AX3 AX4 AX4 AX5 AX5			CitB)	*·
ddh Diaminopimelate Dehydrogenase EC 1.4.1.16 (diaminopimelate dehydrogenase) gluA Glutamate Transport ATP- binding Protein (glutamate-binding protein) gluC Glutamate Transport Permease (glutamate transport Permease (glutamate transport Permease) gluD Glutamate Transport System permease) gluD Glutamate transport System permease) gluA Glycine Hydroxymethyltransferase EC 2.1.2.1 Ishino et al., Nucleic Acids Research 15: 3917 AX1 (1987) EP1108790 AX1 (Fronemeyer et al., Journal of Bacteriology (177(5):1152-8 (1995)) Kronemeyer et al., Journal of Bacteriology (177(5):1152-8 (1995)) Kronemeyer et al., Journal of Bacteriology (177(5):1152-8 (1995)) Kronemeyer et al., X8 Journal of Bacteriology (177(5):1152-8 (1995)) Kronemeyer et al., X8 Journal of Bacteriology (177(5):1152-8 (1995)) Kronemeyer et al., X8 Journal of Bacteriology (177(5):1152-8 (1995)) Kronemeyer et al., X8 Journal of Bacteriology (177(5):1152-8 (1995)) Kronemeyer et al., X8 Journal of Bacteriology (177(5):1152-8 (1995)) Kronemeyer et al., X8 Journal of Bacteriology (177(5):1152-8 (1995)) Kronemeyer et al., X8 Journal of Bacteriology (177(5):1152-8 (1995)) Kronemeyer et al., X8 Journal of Bacteriology (177(5):1152-8 (1995)) Kronemeyer et al., X8 Journal of Bacteriology (177(5):1152-8 (1995)) Kronemeyer et al., X8 Journal of Bacteriology (177(5):1152-8 (1995)) Kronemeyer et al., X8 Journal of Bacteriology (177(5):1152-8 (1995)) Kronemeyer et al., X8 Journal of Bacteriology (177(5):1152-8 (1995)) Kronemeyer et al., X8 Journal of Bacteriology (177(5):1152-8 (1995)) Kronemeyer et al., X8 Journal of Bacteriology (177(5):1152-8 (1995)) Kronemeyer et al., X8 Journal of Bacteriology (177(5):1152-8 (1995))	AX065421		<u> </u>	citE
ddh Diaminopimelate Dehydrogenase EC 1.4.1.16 (diaminopimelate dehydrogenase) gluA Glutamate Transport ATP-binding Protein (glutamate-binding protein) gluB Glutamate Transport ATP-binding Protein (glutamate-binding protein) gluC Glutamate Transport ATP-permease (glutamate transport system permease) gluD Glutamate Transport System permease) gluD Glutamate transport System permease) glyA Glycine Hydroxymethyltransferase EC 2.1.2.1	AX127146	EP1108790		
Dehydrogenase EC 1.4.1.16 (diaminopimelate dehydrogenase) gluA Glutamate Transport ATP- binding Protein (glutamate-binding Protein) (glutamate-binding protein) gluC Glutamate Transport Permease (glutamate transport Permease (glutamate transport System permease) gluD Glutamate Transport Permease (glutamate Transport System permease) gluD Glutamate transport System permease) gluC Glutamate Transport System permease) gluD Glutamate Transport System permease) gluD Glutamate Transport System permease glyA Glycine System permease GlyCine System Permease GlyCine System Permease GlyCine System Permease				
EC 1.4.1.16 (diaminopimelate dehydrogenase) gluA Glutamate Transport ATP-binding Protein (glutamate-binding Protein (glutamate-binding protein) gluB Glutamate-binding Protein (glutamate-binding protein) gluC Glutamate Transport Permease (glutamate transport Permease (glutamate transport System permease) gluD Glutamate Transport Permease (glutamate transport System permease) gluC Glutamate Transport Reonemeyer et al., Journal of Bacteriology (177(5):1152-8 (1995)) gluC Glutamate Transport Reonemeyer et al., Journal of Bacteriology (177(5):1152-8 (1995)) gluD Glutamate Transport Reonemeyer et al., Journal of Bacteriology (177(5):1152-8 (1995)) gluC Glutamate Transport Reonemeyer et al., Journal of Bacteriology (177(5):1152-8 (1995)) gluC Glutamate Transport Reonemeyer et al., Journal of Bacteriology (177(5):1152-8 (1995)) gluC Glutamate Transport Reonemeyer et al., Journal of Bacteriology (177(5):1152-8 (1995)) gluC Glutamate Transport Reonemeyer et al., Journal of Bacteriology (177(5):1152-8 (1995)) gluC Glutamate Transport Reonemeyer et al., Journal of Bacteriology (177(5):1152-8 (1995)) gluC Glutamate Transport Reonemeyer et al., Journal of Bacteriology (177(5):1152-8 (1995)) gluC Glutamate Transport Reonemeyer et al., Journal of Bacteriology (177(5):1152-8 (1995)) gluC Glutamate Transport Reonemeyer et al., Journal of Bacteriology (177(5):1152-8 (1995)) gluC Glutamate Transport Reonemeyer et al., Journal of Bacteriology (177(5):1152-8 (1995)) gluC Glutamate Transport Reonemeyer et al., Journal of Bacteriology (177(5):1152-8 (1995)) gluC Glutamate Transport Reonemeyer et al., Journal of Bacteriology (177(5):1152-8 (1995)) gluC Glutamate Transport Reonemeyer et al., Journal of Bacteriology (177(5):1152-8 (1995))	S07384	•	•	ddh
(diaminopimelate dehydrogenase) gluA Glutamate Transport ATP- binding Protein (glutamate transport ATP- binding protein) gluB Glutamate-binding Protein (glutamate-binding protein) gluC Glutamate Transport ATP- bermease (glutamate transport System permease) gluD Glutamate Transport (glutamate transport System permease) gluD Glycine (glutamate Transport System permease) glyA Glycine (Hydroxymethyltransferase) glyA Glycine (Hydroxymethyltransferase) EC 2.1.2.1	AX127152		1 -	
dehydrogenase) gluA Glutamate Transport ATP- binding Protein (glutamate transport ATP- binding protein) gluB Glutamate-binding Protein (glutamate-binding protein) gluC Glutamate Transport Permease (glutamate transport Permease (glutamate transport Permease) gluD Glutamate Transport gluD Glutamate Transport permease) gluD Glutamate transport permease (glutamate transport permease) gluD Glutamate transport permease (glutamate transport permease) glyA Glycine Hydroxymethyltransferase EC 2.1.2.1				
gluA Glutamate Transport ATP- binding Protein (glutamate transport ATP- binding protein) gluB Glutamate-binding Protein (glutamate-binding protein) gluC Glutamate Transport Permease (glutamate transport System permease) gluD Glutamate Transport Permease (glutamate transport System permease) gluD Glutamate transport System permease) gluA Glycine Hydroxymethyltransferase EC 2.1.2.1 Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995) Kronemeyer et al., AX8 AX0 AX0 AX0		EP1108/90		
binding Protein (glutamate transport ATP- binding protein) gluB Glutamate-binding Protein (glutamate-binding protein) gluC Glutamate Transport Permease (glutamate transport protein) gluD Glutamate transport Permease (glutamate transport protein) glutamate transport glutamate transport glutamate transport glutamate transport glutamate transport glutamate transport permease (glutamate transport permease (gluta	7.01101		· 	
(glutamate transport ATP-binding protein) gluB Glutamate-binding Protein (glutamate-binding protein) gluC Glutamate Transport Kronemeyer et al., Permease (glutamate transport system permease) gluD Glutamate Transport Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995) gluD Glutamate Transport Kronemeyer et al., Journal of Bacteriology (glutamate Transport Formease (glutamate transport System permease) gluD Glutamate Transport Kronemeyer et al., Journal of Bacteriology (glutamate transport System permease) glyA Glycine W00109843 AX0 AX0 AF3	X81191	-	_	grua
binding protein) gluB Glutamate-binding Protein (glutamate-binding protein) gluC Glutamate Transport Kronemeyer et al., Y8 Permease Journal of Bacteriology 177(5):1152-8 (1995) gluC Glutamate Transport Kronemeyer et al., Y8 Permease Journal of Bacteriology 177(5):1152-8 (1995) system permease) gluD Glutamate Transport Kronemeyer et al., Y8 Permease Journal of Bacteriology 177(5):1152-8 (1995) system permease) glyA Glycine W00109843 Ax0 Hydroxymethyltransferase EC 2.1.2.1		<u> </u>	<u> </u>	
GluB Glutamate-binding Protein (glutamate-binding protein) 177(5):1152-8 (1995)		1//(3):1132-0 (1993)	1 17 .	
(glutamate-binding protein) gluC Glutamate Transport Rronemeyer et al., yermease Journal of Bacteriology 177(5):1152-8 (1995) gluC Glutamate Transport Rronemeyer et al., yermease Journal of Bacteriology 177(5):1152-8 (1995) gluD Glutamate Transport Rronemeyer et al., yermease Journal of Bacteriology 177(5):1152-8 (1995) gluC Glutamate transport Rronemeyer et al., yermease Journal of Bacteriology 177(5):1152-8 (1995) glyA Glycine W00109843 Ax0 Hydroxymethyltransferase EC 2.1.2.1	X81191	Examonar of al		-772
protein) gluC Glutamate Transport Permease (glutamate transport system permease) gluD Glutamate Transport Permease (glutamate Transport Kronemeyer et al., X8 Permease (glutamate Transport Kronemeyer et al., X8 Permease (glutamate transport Journal of Bacteriology (glutamate transport 177(5):1152-8 (1995) system permease) glyA Glycine Hydroxymethyltransferase EC 2.1.2.1	VOTIBI	_	_	grap
Glutamate Transport Permease (glutamate transport system permease) GluD Glutamate Transport Permease (glutamate Transport Permease (glutamate transport System permease) GlyA Glycine Hydroxymethyltransferase EC 2.1.2.1 Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995) Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995) WO0100843 AXO AF3			<u> </u>	
Permease (glutamate transport system permease) gluD Glutamate Transport Kronemeyer et al., Journal of Bacteriology (glutamate Transport Journal of Bacteriology (glutamate transport 177(5):1152-8 (1995) system permease) glyA Glycine Hydroxymethyltransferase EC 2.1.2.1	X81191			~711C
(glutamate transport system permease) GluD Glutamate Transport Kronemeyer et al., X8 Permease Journal of Bacteriology (glutamate transport system permease) GlyA Glycine W00100843 Hydroxymethyltransferase EC 2.1.2.1	VOTTOT		_	grac
system permease) gluD Glutamate Transport Kronemeyer et al., X8 Permease Journal of Bacteriology (glutamate transport 177(5):1152-8 (1995) system permease) glyA Glycine W00100843 AX0 Hydroxymethyltransferase EC 2.1.2.1				İ
Glutamate Transport Permease (glutamate transport system permease) GlyA Glycine Hydroxymethyltransferase EC 2.1.2.1 Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995) W00100843 AX0 AF3		1,,(3),1132 6 (1333)	_	Ì
Permease Journal of Bacteriology (glutamate transport 177(5):1152-8 (1995) system permease) GlyA Glycine W00100843 AX0 Hydroxymethyltransferase EC 2.1.2.1	X81191	Kronemever et al		alun
(glutamate transport 177(5):1152-8 (1995) system permease) GlyCine W00100843 AX0 Hydroxymethyltransferase EC 2.1.2.1	1102202	_	-	grub
system permease) glyA Glycine W00100843 AX0 Hydroxymethyltransferase AF3		- -	1 _	Ì
glyA Glycine W00100843 AX0 Hydroxymethyltransferase EC 2.1.2.1			1	i
Hydroxymethyltransferase EC 2.1.2.1 AF3	AX063861	WO0100843		alva
EC 2.1.2.1	AF327063	1	1 -	3-74
				}
I I I MALVELLICE I			(glycine	1
hydroxymethyltransferase)				}
	A47044	Möckel et al. Journal	المراجعة المراجعة المراجعة المراجعة المراجعة المراجعة المراجعة المراجعة المراجعة المراجعة المراجعة المراجعة الم	ilva
	L01508		•	
1 1 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2	AX127150			}

		EP1108790	
<u></u> _/			
luxR	Transcription Regulator	WO0100842	AX065953
	LuxR	EP1108790	AX123320
	(transcription regulator		
	LuxR)		
lysR1	Transcription Regulator	EP1108790	AX064673
	LysR1		AX127144
	(transcription regulator		
	LysR1)		
lysR2	Transcription Activator	EP1108790	AX123312
	LysR2	-	
	(transcription regulator		
	LysR2)		
lysR3	Transcription Regulator	WO0103842	AX065957
	LysR3	EP1108790	AX127150
	(transcription regulator		
 	LysR3)		**06500
panB	Ketopantoate	US6177264	x96580
	Hydroxymethyltransferase		
	EC 2. 1. 2. 11		
•	(ketopantoate	•	
	hydroxymethyltransferase)	***************************************	VOCEON
panC	Pantothenate Synthetase	US6177264	x96580
	EC 6.3.2.1		
	(pantothenate synthetase)	***************************************	AX064959
poxB	Pyruvate Oxidase	WO0100844	AX137665
	EC 1.2.3.3	EP1096013	WYT2 1003
 	(pyruvate oxidase)	771106602	AX113822
zwa2	Cell Growth Factor 2	EP1106693	AX113622 AX127146
	(growth factor 2)	EP1108790	<u> </u>

The invention accordingly also provides a process for the production of coryneform bacteria which produce L-valine, which comprises

- isolating the nucleotide sequence of at least one desired ORF, gene or allele of valine production, optionally including the expression and/or regulation signals,
- b) providing the 5' and the 3' end of the ORF, gene or allele with nucleotide sequences of the target site,
 - c) preferably incorporating the nucleotide sequence of the desired ORF, gene or allele provided with nucleotide sequences of the target site into a vector

which does not replicate or replicates to only a limited extent in coryneform bacteria,

- transferring the nucleotide sequence according to b) d) or c) into coryneform bacteria, and
- 5 isolating coryneform bacteria in which the nucleotide e) sequence according to a) is incorporated at the target site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables 10 transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the target site.

15

25

During work on the present invention, it was possible to incorporate a second copy of an lysCFBR allele into the gluB gene of Corynebacterium glutamicum such that no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remained at the gluB gene 20 site. This strain, which is called DSM13994glu::lysC carries the lysCFBR allele lysC T311I at its natural lysC site and a second copy of the lysCFBR allele lysC T311I at a second site (target site), namely the gluB gene. A plasmid with the aid of which the incorporation of the lysCFBR allele into the gluB gene can be achieved is shown in Figure 1. It carries the name pK18mobsacBglu1_1.

During work on the present invention, it was furthermore possible to incorporate a copy of an lysCFBR allele into the target site of the gluB gene of Corynebacterium glutamicum such that no nucleotide sequence which is capable 30 of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remained at the gluB gene site.

This strain, which is called DSM12866glu::lysC, carries the wild-type form of the lysC gene at its natural lysC site and a second copy of the lysC gene in the form of the lysCFBR allele lysC T311I at a second site (target site), namely the gluB gene. It has been deposited under number DSM15039 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures). A plasmid with the aid of which the incorporation of the lysCFBR allele into the gluB gene can be achieved is shown in Figure 1. It carries the name

51

PCT/EP02/08464

WO 03/040373

pK18mobsacBglu1_1.

10

30

35

During work on the present invention, it was furthermore possible to incorporate a copy of an lysCFBR allele into the target site of the aecD gene of Corynebacterium glutamicum 15 such that no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remained at the aecD gene site. This strain, which is called DSM12866aecD::lysC, carries 20 the wild-type form of the lysC gene at its natural lysC site and a second copy of the lysC gene in the form of the lysCFBR allele lysC T311I at a second site (target site), namely the aecD gene. A plasmid with the aid of which the incorporation of the lysCFBR allele into the aecD gene can 25 be achieved is shown in Figure 2. It carries the name pK18mobsacBaecD1_1.

During work on the present invention, it was furthermore possible to incorporate a copy of an lysCFBR allele into the target site of the pck gene of Corynebacterium glutamicum such that no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remained at the pck gene site.

WO 03/040373 PCT/EP02/08464 52

This strain, which is called DSM12866pck::lysC, carries the wild-type form of the lysC gene at its natural lysC site and a second copy of the lysC gene in the form of the lysC^{FBR} allele lysC T311I at a second site (target site), namely the pck gene. A plasmid with the aid of which the incorporation into the pck gene can be achieved is shown in Figure 3. It carries the name pK18mobsacBpck1_1.

During work on the present invention, it was furthermore possible to incorporate a copy of the ddh gene into the target site of the gluB gene of Corynebacterium glutamicum 10 such that no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remained at the gluB gene site. 15 This strain, which is called DSM12866glu::ddh, carries a copy of the ddh gene at its natural ddh site and a second copy of the ddh gene at a second site (target site), namely the gluB gene. A plasmid with the aid of which the incorporation of the ddh gene into the gluB gene can be 20 achieved is shown in Figure 4. It carries the name pK18mobsacBgluB2_1.

During work on the present invention, it was furthermore possible to incorporate a copy of the dapA gene into the target site of the aecD gene of Corynebacterium glutamicum such that no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remained at the aecD gene site. This strain, which is called DSM12866aecD::dapA, carries a copy of the dapA gene at its natural dapA site and a second copy of the dapA gene at a second site (target site), namely the aecD gene. A plasmid with the aid of which the incorporation of the dapA gene into the aecD gene can be

25

30

35

achieved is shown in Figure 5. It carries the name pK18mobsacBaecD2_1.

During work on the present invention, it was furthermore possible to incorporate a copy of a pyc allele into the target site of the pck gene of Corynebacterium glutamicum such that no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remained at the pck gene site. 10 This strain, which is called DSM12866pck::pyc, carries a copy of the wild-type form of the pyc gene at its natural pyc site and a second copy of the pyc gene in the form of the pyc allele pyc P458S at a second site (target site), namely the pck gene. A plasmid with the aid of which the 15 incorporation of the pyc allele into the pck gene can be achieved is shown in Figure 6. It carries the name pK18mobsacBpck1_3.

The coryneform bacteria produced according to the invention

20 can be cultured continuously or discontinuously in the
batch process (batch culture) or in the fed batch (feed
process) or repeated fed batch process (repetitive feed
process) for the purpose of production of chemical
compounds. A summary of known culture methods is described

25 in the textbook by Chmiel (Bioprozesstechnik 1. Einführung
in die Bioverfahrenstechnik (Gustav Fischer Verlag,
Stuttgart, 1991)) or in the textbook by Storhas
(Bioreaktoren und periphere Einrichtungen (Vieweg Verlag,
Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology

(Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid or lactic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e. g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the above-mentioned substances. Suitable precursors can moreover be added to the culture

20

25

30

35

medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, such as e.g.

reached within 10 hours to 160 hours.

antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 20°C to 45°C, and preferably 25°C to 40°C. Culturing is continued until a maximum of the desired

PCT/EP02/08464

It has been found that the coryneform bacteria according to the invention, in particular the coryneform bacteria which produce L-lysine, have an unexpectedly high stability. They were stable for at least 10-20, 20-30, 30-40, 40-50, preferably at least 50-60, 60-70, 70-80 and 80-90 generations or cell division cycles.

chemical compound has formed. This target is usually

15 The following microorganisms have been deposited:

20

The strain Corynebacterium glutamicum DSM12866glu::lysC was deposited in the form of a pure culture on 5th June 2002 under number DSM15039 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty.

The plasmid pK18mobsacBglu1_1 was deposited in the form of a pure culture of the strain E. coli
DH5cmcr/pK18mobsacBglu1_1 (=

DH5alphamcr/pK18mobsacBglu1_1) on 20th April 2001 under number DSM14243 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

The plasmid pK18mobsacBaecD1_1 was deposited in the form of a pure culture of the strain E. coli

DH50mcr/pK18mobsacBaecD1_1 (=

DH5alphamcr/pK18mobsacBaecD1_1) on 5th June 2002 under number DSM15040 at the Deutsche Sammlung für

Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

Example 1

Incorporation of a second copy of the lysC^{FBR} allele into the chromosome of the strain DSM13994 and of the strain DSM12866

The Corynebacterium glutamicum strain DSM13994 was produced by multiple, non-directed mutagenesis, selection and mutant selection from C. glutamicum ATCC13032. The strain is resistant to the lysine analogue S-(2-aminoethyl)-L-10 cysteine and has a feed back-resistant aspartate kinase which is insensitive to inhibition by a mixture of lysine and threonine (in each case 25 mM). The nucleotide sequence of the lysCFBR allele of this strain is shown as SEQ ID NO:3. It is also called lysC T311I in the following. The amino acid sequence of the aspartate kinase protein coded is shown as SEQ ID NO:4. A pure culture of this strain was deposited on 16th January 2001 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty.

The strain DSM12866 was produced from C. glutamicum ATCC13032 by non-directed mutagenesis and selection of the mutants with the best L-lysine accumulation. It is

25 methionine-sensitive. Growth on minimal medium comprising L-methionine can be re-established by addition of threonine. This strain has the wild-type form of the lysC gene shown as SEQ ID NO:1. The corresponding amino acid sequence of the wild-type aspartate kinase protein is shown as SEQ ID NO:2. A pure culture of this strain was deposited on 10th June 1999 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

10

1.1 Isolation and sequencing of the DNA of the lysC allele of strain DSM13994

From the strain DSM13994, chromosomal DNA is isolated by the conventional methods (Eikmanns et al., Microbiology 140: 1817 - 1828 (1994)). With the aid of the polymerase chain reaction, a DNA section which carries the lysC gene or allele is amplified. On the basis of the sequence of the lysC gene known for C. glutamicum (Kalinowski et al., Molecular Microbiology, 5 (5), 1197 - 1204 (1991); Accession Number X57226), the following primer oligonucleotides were chosen for the PCR:

lysClbeg (SEQ ID No: 5):
5 TA(G GAT CC)T CCG GTG TCT GAC CAC GGT G 3

lysC2end: (SEQ ID NO: 6):

15 5' AC(G GAT CC)G CTG GGA AAT TGC GCT CTT CC 3'

The primers shown are synthesized by MWG Biotech and the PCR reaction is carried out by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers allow amplification of a DNA section of approx. 1.7 kb in length, which carries the lysC gene or allele. The primers moreover contain the sequence for a cleavage site of the restriction endonuclease BamHI, which is marked by parentheses in the nucleotide sequence shown above.

- The amplified DNA fragment of approx. 1.7 kb in length which carries the lysC allele of the strain DSM13994 is identified by electrophoresis in a 0.8% agarose gel, isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).
- Ligation of the fragment is then carried out by means of the Topo TA Cloning Kit (Invitrogen, Leek, The Netherlands, Cat. Number K4600-01) in the vector pCRII-TOPO. The ligation batch is transformed in the E. coli strain TOP10

(Invitrogen, Leek, The Netherlands). Selection of plasmidcarrying cells is made by plating out the transformation batch on kanamycin (50 mg/l)-containing LB agar with X-Gal $(5-bromo-4-chloro-3-indolyl \beta-D-galactopyranoside,$ 64 mg/l).

The plasmid obtained is checked by means of restriction cleavage, after isolation of the DNA, and identified in agarose gel. The resulting plasmid is called pCRIITOPOlysC.

The nucleotide sequence of the amplified DNA fragment or PCR product is determined by the dideoxy chain termination 10 method of Sanger et al. (Proceedings of the National Academy of Sciences USA, 74:5463-5467 (1977)) using the "ABI Prism 377" sequencing apparatus of PE Applied Biosystems (Weiterstadt, Germany). The sequence of the coding region of the PCR product is shown in SEQ ID No:3. 15 The amino acid sequence of the associated aspartate kinase protein is shown in SEQ ID NO:4.

The base thymine is found at position 932 of the nucleotide sequence of the coding region of the lysCFBR allele of 20 strain DSM13994 (SEQ ID NO:3). The base cytosine is found at the corresponding position of the wild-type gene (SEQ ID NO:1).

The amino acid isoleucine is found at position 311 of the amino acid sequence of the aspartate kinase protein of strain DSM13994 (SEQ ID No:4). The amino acid threonine is found at the corresponding position of the wild-type protein (SEQ ID No:2).

25

30

The lysC allele, which contains the base thymine at position 932 of the coding region and accordingly codes for an aspartate kinase protein which contains the amino acid isoleucine at position 311 of the amino acid sequence, is called the lysCFBR allele or lysC T311I in the following.

The plasmid pCRIITOPOlysC, which carries the lysCFBR allele lysC T311I, was deposited in the form of a pure culture of the strain E. coli TOP 10/pCRIITOPOlysC under number DSM14242 on 20th April 2001 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

1.2 Construction of the replacement vector pK18mobsacBglu1_1

The Corynebacterium glutamicum strain ATCC13032 is used as the donor for the chromosomal DNA. From the strain 10 ATCC13032, chromosomal DNA is isolated using the conventional methods (Eikmanns et al., Microbiology 140: 1817 - 1828 (1994)). With the aid of the polymerase chain reaction, a DNA fragment which carries the gluB gene and surrounding regions is amplified. On the basis of the 15 sequence of the gluABCD gene cluster known for C. glutamicum (Kronemeyer et al., Journal of Bacteriology, 177: 1152 - 1158 (1995)) (Accession Number X81191), the following primer oligonucleotides are chosen for the PCR:

gluBgl1 (SEQ ID NO: 7): 5 TA (A GAT CT) G TGT TGG ACG TCA TGG CAA G 3 gluBgl2 (SEQ ID NO: 8): 5 AC (A GAT CT) T GAA GCC AAG TAC GGC CAA G 3

The primers shown are synthesized by MWG Biotech and the PCR reaction is carried out by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers allow amplification of a DNA fragment of approx 1.7 kb in size, which carries the glub gene and surrounding regions. The surrounding regions are a sequence section approx. 0.33 kb 30 in length upstream of the gluB gene, which represents the 3' end of the gluA gene, and a sequence section approx. 0.44 kb in length downstream of the gluB gene, which

represents the 5' end of the gluC gene. The primers moreover contain the sequence for the cleavage site of the restriction endonuclease BglII, which is marked by parentheses in the nucleotide sequence shown above.

- The amplified DNA fragment of approx. 1.7 kb in length which carries the gluB gene and surrounding regions is identified by means of electrophoresis in a 0.8% agarose gel and isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).
- Ligation of the fragment is then carried out by means of the TOPO TA Cloning Kit (Invitrogen, Leek, The Netherlands, Cat. Number K4600-01) in the vector pCRII-TOPO. The ligation batch is transformed in the E. coli strain TOP10 (Invitrogen, Leek, The Netherlands). Selection of plasmid-carrying cells is made by plating out the transformation batch on kanamycin (50 mg/l)-containing LB agar with X-Gal (5-bromo-4-chloro-3-indolyl β -D-galactopyranoside, 64 mg/l).

The plasmid obtained is checked by means of restriction cleavage, after isolation of the DNA, and identified in agarose gel. The resulting plasmid is called pCRII-TOPOglu.

The plasmid pCRII-TOPOglu is cleaved with the restriction enzyme BglII (Amersham-Pharmacia, Freiburg, Germany) and after separation in an agarose gel (0.8%) with the aid of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) the gluB fragment of approx. 1.7 kb is isolated from the agarose gel and employed for ligation with the mobilizable cloning vector pK18mobsacB described by Schäfer et al. (Gene 14: 69-73 (1994)). This is cleaved beforehand with the restriction enzyme BamHI and dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim), mixed with the gluB fragment of approx. 1.7 kb, and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).

- The E. coli strain DH5 α (Grant et al.; Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) is then transformed with the ligation batch (Hanahan, In. DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which is supplemented with 50 mg/l kanamycin.
- Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage and subsequent agarose gel electrophoresis. The plasmid is called pK18mobsacBglu1.
- Plasmid DNA was isolated from the strain DSM14242 (see Example 1.1), which carries the plasmid pCRIITOPOlysC, and cleaved with the restriction enzyme BamHI (Amersham-Pharmacia, Freiburg, Germany), and after separation in an agarose gel (0.8%) with the aid of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) the lysCFBR-
- containing DNA fragment of approx. 1.7 kb in length was isolated from the agarose gel and employed for ligation with the vector pK18mobsacBglu1 described above. This is cleaved beforehand with the restriction enzyme BamHI, dephosphorylated with alkaline phosphatase (Alkaline)
- Phosphatase, Boehringer Mannheim, Germany), mixed with the lysC^{FBR} fragment of approx. 1.7 kb and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).
- The E. coli strain DH50mcr (Life Technologies GmbH,

 30 Karlsruhe, Germany) is then transformed with the ligation batch (Hanahan, In: DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989).

 Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al.,

 35 Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold

Spring Harbor, New York, 1989), which was supplemented with 50 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage and subsequent agarose gel electrophoresis. The plasmid is called pK18mobsacBglu1_1. A map of the plasmid is shown in Figure 1.

The plasmid pK18mobsacBglu1_1 was deposited in the form of a pure culture of the strain E. coli

- DH5cmcr/pK18mobsacBglu1_1 (=
 DH5alphamcr/pK18mobsacBglu1_1) under number DSM14243 on
 20.04.2001 at the Deutsche Sammlung für Mikroorganismen und
 Zellkulturen (DSMZ, Braunschweig, Germany) in accordance
 with the Budapest Treaty.
- 15 1.3 Incorporation of a second copy of the lysC^{FBR} allele lysC T311I into the chromosome (target site: gluB gene) of the strain DSM13994 by means of the replacement vector pK18mobsacBglu1_1

The vector pK18mobsacBglu1_1 described in Example 1.2 is transferred by the protocol of Schäfer et al. (Journal of 20 Microbiology 172: 1663-1666 (1990)) into the C. glutamicum strain DSM13994 by conjugation. The vector cannot replicate independently in DSM13994 and is retained in the cell only if it has integrated into the chromosome. Selection of clones or transconjugants with integrated pK18mobsacBglu1_1 25 is made by plating out the conjugation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which is supplemented with 15 mg/l kanamycin and 50 mg/l nalidixic acid. Kanamycin-resistant transconjugants are plated out on 30 LB agar plates with 25 mg/l kanamycin and incubated for 48 hours at 33°C.

5

For selection of mutants in which excision of the plasmid has taken place as a consequence of a second recombination event, the clones are cultured for 20 hours in LB liquid medium and then plated out on LB agar with 10% sucrose and incubated for 48 hours.

The plasmid pK18mobsacBglu1_1, like the starting plasmid pK18mobsacB, contains, in addition to the kanamycin resistance gene, a copy of the sacB gene which codes for levan sucrase from Bacillus subtilis. The expression which can be induced by sucrose leads to the formation of levan sucrase, which catalyses the synthesis of the product levan, which is toxic to C. glutamicum. Only those clones in which the integrated pK18mobsacBglu1_1 has excised as the consequence of a second recombination event therefore grow on LB agar. Depending on the position of the second recombination event, after the excision the second copy of the lysCFBR allele manifests itself in the chromosome at the gluB locus, or the original gluB locus of the host remains.

Approximately 40 to 50 colonies are tested for the

20 phenotype "growth in the presence of sucrose" and "nongrowth in the presence of kanamycin". Approximately 20
colonies which show the phenotype "growth in the presence
of sucrose" and "non-growth in the presence of kanamycin"
are investigated with the aid of the polymerase chain

25 reaction. A DNA fragment which carries the gluB gene and
surrounding regions is amplified here from the chromosomal
DNA of the colonies. The same primer oligonucleotides as
are described in Example 1.2 for the construction of the
integration plasmid are chosen for the PCR.

30 gluBgl1 (SEQ ID NO: 7):

5` TA(A GAT CT)G TGT TGG ACG TCA TGG CAA G 3`

gluBgl2 (SEQ ID NO: 8):

5` AC(A GAT CT)T GAA GCC AAG TAC GGC CAA G 3`

15

The primers allow amplification of a DNA fragment approx.

1.7 kb in size in control clones with the original gluB locus. In clones with a second copy of the lysc allele in the chromosome at the gluB locus, DNA fragments with a size of approx. 3.4 kb are amplified.

The amplified DNA fragments are identified by means of electrophoresis in a 0.8% agarose gel.

A clone which, in addition to the copy present at the lysC locus, has a second copy of the lysC^{FRB} allele lysC T311I at the gluB locus in the chromosome was identified in this manner. This clone was called strain DSM13994glu::lysC.

1.4 Incorporation of a second copy of the lysC gene in the form of the lysC^{FBR} allele lysC T311I into the chromosome (target site: gluB gene) of the strain DSM12866 by means of the replacement vector pK18mobsacBglu1_1

As described in Example 1.3, the plasmid pK18mobsacBglu1_1 is transferred into the C. glutamicum strain DSM12866 by conjugation. A clone which, in addition to the copy of the wild-type gene present at the lysC locus, has a second copy of the lysC gene in the form of the lysC^{FBR} allele lysC T311I at the gluB locus in the chromosome was identified in the manner described in 1.3. This clone was called strain DSM12866glu::lysC.

The Corynebacterium glutamicum strain according to the invention which carries a second copy of an lysCFBR allele in the gluB gene was deposited in the form of a pure culture of the strain Corynebacterium glutamicum DSM12866glu::lysC on 5th June 2002 under number DSM15039 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

1.5 'Construction of the replacement vector pK18mobsacBpck1_1

The Corynebacterium glutamicum strain ATCC13032 is used as the donor for the chromosomal DNA. From the strain

5 ATCC13032, chromosomal DNA is isolated using the conventional methods (Eikmanns et al., Microbiology 140: 1817 - 1828 (1994)). With the aid of the polymerase chain reaction, a DNA fragment which carries the pck gene and surrounding regions is amplified. On the basis of the sequence of the pck gene known for C. glutamicum (EP1094111 and Riedel et al., Journal of Molecular and Microbiological Biotechnology 3:573-583 (2001)) (Accession Number AJ269506), the following primer oligonucleotides are chosen for the PCR:

pck_beg (SEQ ID NO: 9):

5` TA(A GAT CT) G CCG GCA TGA CTT CAG TTT 3`

pck_end (SEQ ID NO: 10):

5 AC (A GAT CT) G GTG GGA GCC TTT CTT GTT ATT3 `

The primers shown are synthesized by MWG Biotech and the
20 PCR reaction is carried out by the standard PCR method of
Innis et al. (PCR Protocols. A Guide to Methods and
Applications, 1990, Academic Press). The primers allow
amplification of a DNA fragment of approx 2.9 kb in size,
which carries the pck gene and adjacent regions. The
25 primers moreover contain the sequence for the cleavage site
of the restriction endonuclease BglII, which is marked by
parentheses in the nucleotide sequence shown above.

The amplified DNA fragment of approx. 2.9 kb in length which carries the pck gene and surrounding regions is identified by means of electrophoresis in a 0.8% agarose gel and isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

Ligation of the fragment is then carried out by means of the TOPO TA Cloning Kit (Invitrogen, Leek, The Netherlands, Cat. Number K4600-01) in the vector pCRII-TOPO. The ligation batch is transformed in the E. coli strain TOP10 (Invitrogen, Leek, The Netherlands). Selection of plasmid-carrying cells is made by plating out the transformation batch on kanamycin (50 mg/l)-containing LB agar with X-Gal (64 mg/l).

The plasmid obtained is checked by means of restriction cleavage, after isolation of the DNA, and identified in agarose gel. The resulting plasmid is called pCRII-TOPOpck.

15

20

The plasmid pCRII-TOPOpck is cleaved with the restriction enzyme BglII (Amersham-Pharmacia, Freiburg, Germany) and after separation in an agarose gel (0.8%) with the aid of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) the pck fragment of approx. 2.9 kb is isolated from the agarose gel and employed for ligation with the mobilizable cloning vector pK18mobsacB described by Schäfer et al. (Gene 14: 69-73 (1994)). This is cleaved beforehand with the restriction enzyme BamHI and dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim), mixed with the pck fragment of approx. 2.9 kb, and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli Strain DH5α (Grant et al.; Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) is then transformed with the ligation batch (Hanahan, In. DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989) Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which is supplemented with 50 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage and subsequent agarose gel electrophoresis. The plasmid is called pK18mobsacBpck1.

- Plasmid DNA was isolated from the strain DSM14242 (see Example 1.1), which carries the plasmid pCRIITOPOlysC, and cleaved with the restriction enzyme BamHI (Amersham-Pharmacia, Freiburg, Germany), and after separation in an agarose gel (0.8%) with the aid of the QIAquick Gel
- 10 Extraction Kit (Qiagen, Hilden, Germany) the lysCFBR_containing DNA fragment approx. 1.7 kb long was isolated from the agarose gel and employed for ligation with the vector pK18mobsacBpckl described above. This is cleaved beforehand with the restriction enzyme BamHI,
- dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim, Germany), mixed with the lysCFBR fragment of approx. 1.7 kb and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).
- The E. coli strain DH50mcr (Life Technologies GmbH,
 Karlsruhe, Germany) is then transformed with the ligation
 batch (Hanahan, In: DNA Cloning. A Practical Approach. Vol.
 1, ILR-Press, Cold Spring Harbor, New York, 1989).
 Selection of plasmid-carrying cells is made by plating out
 the transformation batch on LB agar (Sambrook et al.,
 Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold
 Spring Harbor, New York, 1989), which was supplemented with
 50 mg/l kanamycin.
- Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage and subsequent agarose gel electrophoresis. The plasmid is called pK18mobdsacBpck1_1.

 A map of the plasmid is shown in Figure 3.

5

WO 03/040373 PCT/EP02/08464 **68**

Incorporation of a second copy of the lysC gene in 1.6 the form of the lysCFBR allele lysC T311I into the chromosome (target site: pck gene) of the strain DSM12866 by means of the replacement vector pK18mobsacBpck1_1

As described in Example 1.3, the plasmid pK18mobsacBpck1_1 described in Example 1.5 is transferred into the C. glutamicum strain DSM12866 by conjugation. Selection is made for targeted recombination events in the chromosome of C. glutamicum DSM12866 as described in Example 1.3. 10 Depending on the position of the second recombination event, after the excision the second copy of the lysCFBR allele manifests itself in the chromosome at the pck locus, or the original pck locus of the host remains.

Approximately 40 to 50 colonies are tested for the 15 phenotype "growth in the presence of sucrose" and "nongrowth in the presence of kanamycin". Approximately 20 colonies which show the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin" are investigated with the aid of the polymerase chain 20 reaction. A DNA fragment which carries the pck gene and surrounding regions is amplified here from the chromosomal DNA of the colonies. The same primer oligonucleotides as are described in Example 1.5 for the construction of the 25 integration plasmid are chosen for the PCR.

pck_beg (SEQ ID NO: 9): 5 TA (A GAT CT) G CCG GCA TGA CTT CAG TTT 3 pck_end (SEQ ID NO: 10): 5 AC (A GAT CT) G GTG GGA GCC TTT CTT GTT ATT3 `

The primers allow amplification of a DNA fragment approx. 30 2.9 kb in size in control clones with the original pck locus. In clones with a second copy of the lysCFBR allele in the chromosome at the pck locus, DNA fragments with a size of approx. 4.6 kb are amplified.

The amplified DNA fragments are identified by means of electrophoresis in a 0.8% agarose gel.

- 5 A clone which, in addition to the copy of the wild-type gene present at the lysC locus, has a second copy of the lysC gene in the form of the lysC^{FBR} allele lysC T311I at the pck locus in the chromosome was identified in this manner. This clone was called strain DSM12866pck::lysC.
- 10 1.7 Construction of the replacement vector pK18mobsacBaecD1_1

The Corynebacterium glutamicum strain ATCC13032 is used as the donor for the chromosomal DNA. From the strain ATCC13032, chromosomal DNA is isolated using the conventional methods (Eikmanns et al., Microbiology 140: 1817 - 1828 (1994)). With the aid of the polymerase chain reaction, a DNA fragment which carries the aecD gene and surrounding regions is amplified. On the basis of the sequence of the aecD gene known for C. glutamicum (Rossol et al., Journal of Bacteriology 174:2968-2977 (1992)) (Accession Number M89931), the following primer oligonucleotides are chosen for the PCR:

aecD_beg (SEQ ID NO: 11):
5` GAA CTT ACG CCA AGC TGT TC 3`

25 aecD_end (SEQ ID NO: 12): 5` AGC ACC ACA ATC AAC GTG AG 3`

30

The primers shown are synthesized by MWG Biotech and the PCR reaction is carried out by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers allow amplification of a DNA fragment of approx 2.1 kb in size, which carries the aecD gene and adjacent regions.

The amplified DNA fragment of approx. 2.1 kb in length is identified by means of electrophoresis in a 0.8% agarose gel and isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

5 The DNA fragment purified is cleaved with the restriction enzyme BamHI and EcoRV (Amersham Pharmacia, Freiburg, Germany). The ligation of the fragment in the vector pUC18 then takes place (Norrander et al., Gene 26:101-106 (1983)). This is cleaved beforehand with the restriction enzymes BglII and SmaI, dephosphorylated, mixed with the 10 aecD-carrying fragment of approx. 1.5 kb, and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany). The ligation batch is transformed in the E. coli strain TOP10 (Invitrogen, Leek, The Netherlands). Selection of plasmid-carrying cells is made 15 by plating out the transformation batch on kanamycin (50 mg/l)-containing LB agar with X-Gal (64 mg/l).

The plasmid obtained is checked by means of restriction cleavage, after isolation of the DNA, and identified in agarose gel. The resulting plasmid is called pUC18aecD.

20

25

30

35

Germany).

Plasmid DNA was isolated from the strain DSM14242 (see Example 1.1) which carries the plasmid pCRIITOPOlysC and cleaved with the restriction enzyme BamHI (Amersham-Pharmacia, Freiburg, Germany) and then treated with Klenow polymerase. After separation in an agarose gel (0.8%) with the aid of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) the lysCFBR-containing DNA fragment approx. 1.7 kb in length is isolated from the agarose gel and employed for ligation with the vector pUC18aecD described above. This is cleaved beforehand with the restriction enzyme StuI, dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim, Germany), mixed with the lysCFBR fragment of approx. 1.7 kb and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg,

The E. coli strain DH50mcr (Life Technologies GmbH, Karlsruhe, Germany) is then transformed with the ligation batch (Hanahan, In: DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989).

- Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which was supplemented with 50 mg/l kanamycin.
- Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage and subsequent agarose gel electrophoresis. The plasmid is called pUC18aecD1.
- The plasmid pUC18aecD1 is cleaved with the restriction enzyme KpnI and then treated with Klenow polymerase. The 15 plasmid is then cleaved with the restriction enzyme SalI (Amersham-Pharmacia, Freiburg, Germany) and after separation in an agarose gel (0.8%) with the aid of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) the fragment of approx. 3.2 kb which carries aecD and lysC is 20 isolated from the agarose gel and employed for ligation with the mobilizable cloning vector pK18mobsacB described by Schäfer et al. (Gene 14: 69-73 (1994)). This is cleaved beforehand with the restriction enzymes SmaI and SalI and dephosphorylated with alkaline phosphatase (Alkaline 25 Phosphatase, Boehringer Mannheim), mixed with the fragment of approx. 3.2 kb which carries aecD and lysC, and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).
- The E. coli strain DH5α (Grant et al.; Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) is then transformed with the ligation batch (Hanahan, In. DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation

WO 03/040373

batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which is supplemented with 50 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage and subsequent agarose gel electrophoresis. The plasmid is called pK18mobsacBaecD1_1. A map of the plasmid is shown in Figure 2.

The plasmid pK18mobsacBaecD1_1 was deposited in the form of a pure culture of the strain E. coli

DH5cmcr/pK18mobsacBaecD1_1 (=

DH5alphamcr/pK18mobsacBaecD1_1) on 5th June 2002 under number DSM15040 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig,

Germany) in accordance with the Budapest Treaty.

- 1.8 Incorporation of a second copy of the lysC gene as the lysC^{FBR} allele into the chromosome (target site: aecD gene) of the strain DSM12866 by means of the replacement vector pK18mobsacBaecD1_1
- As described in Example 1.3, the plasmid pK18mobsacBaecD1_1 described in Example 1.4 is transferred into the C. glutamicum strain DSM12866 by conjugation. Selection is made for targeted recombination events in the chromosome of C. glutamicum DSM12866 as described in Example 1.3.
- Depending on the position of the second recombination event, after the excision the second copy of the lysC allele manifests itself in the chromosome at the aecD locus, or the original aecD locus of the host remains.

Approximately 40 to 50 colonies are tested for the
30 phenotype "growth in the presence of sucrose" and "nongrowth in the presence of kanamycin". Approximately 20
colonies which show the phenotype "growth in the presence
of sucrose" and "non-growth in the presence of kanamycin"

are investigated with the aid of the polymerase chain reaction. A DNA fragment which carries the aecD gene and surrounding regions is amplified here from the chromosomal DNA of the colonies. The same primer oligonucleotides as are described in Example 1.7 for the construction of the integration plasmid are chosen for the PCR.

aecD_beg (SEQ ID NO: 11): 5` GAA CTT ACG CCA AGC TGT TC 3`

aecD_end (SEQ ID NO: 12):

10 5' AGC ACC ACA ATC AAC GTG AG 3'

The primers allow amplification of a DNA fragment approx. 2.1 kb in size in control clones with the original aecD locus. In clones with a second copy of the lysCFBR allele in the chromosome at the aecD locus, DNA fragments with a size of approx. 3.8 kb are amplified.

The amplified DNA fragments are identified by means of electrophoresis in a 0.8% agarose gel.

A clone which, in addition to the copy of the wild-type gene present at the lysC locus, has a second copy of the lysC gene in the form of the lysC allele lysC T311I at the aecD locus in the chromosome was identified in this manner. This clone was called strain DSM12866aecD::lysC.

Example 2

15

20

Incorporation of a second copy of the ddh gene into the chromosome (target site: gluB gene) of the strain DSM12866

2.1 Construction of the replacement vector pK18mobsacBglu2_1

The Corynebacterium glutamicum strain ATCC13032 is used as the donor for the chromosomal DNA. From the strain ATCC13032, chromosomal DNA is isolated using the conventional methods (Eikmanns et al., Microbiology 140:

1817 - 1828 (1994)). With the aid of the polymerase chain reaction, a DNA fragment which carries the gluB gene and surrounding regions is amplified. On the basis of the sequence of the gluABCD gene cluster known for C.

glutamicum (Kronemeyer et al., Journal of Bacteriology, 177: 1152 - 1158 (1995); EP1108790) (Accession Number X81191 and AX127149), the following primer oligonucleotides are chosen for the PCR:

gluA_beg (SEQ ID NO: 13):

10 5' CAC GGT TGC TCA TTG TAT CC 3'

gluD_end (SEQ ID NO: 14):
5 CGA GGC GAA TCA GAC TTC TT 3 `

The primers shown are synthesized by MWG Biotech and the PCR reaction is carried out by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers allow amplification of a DNA fragment of approx 4.4 kb in size, which carries the gluB gene and surrounding regions.

The amplified DNA fragment is identified by means of electrophoresis in a 0.8% agarose gel and isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

Ligation of the fragment is then carried out by means of the TOPO TA Cloning Kit (Invitrogen, Leek, The Netherlands, Cat. Number K4600-01) in the vector pCRII-TOPO. The ligation batch is transformed in the E. coli strain TOP10 (Invitrogen, Leek, The Netherlands). Selection of plasmid-carrying cells is made by plating out the transformation batch on kanamycin (50 mg/l)-containing LB agar with X-Gal (64 mg/l).

The plasmid obtained is checked by means of restriction cleavage, after isolation of the DNA, and identified in

WO 03/040373 PCT/EP02/08464

75

agarose gel. The resulting plasmid is called pCRII-TOPOglu2.

The plasmid pCRII-TOPOglu2 is cleaved with the restriction enzymes EcoRI and SalI (Amersham-Pharmacia, Freiburg,

5 Germany) and after separation in an agarose gel (0.8%) with the aid of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) the gluB fragment of approx. 3.7 kb is isolated from the agarose gel and employed for ligation with the mobilizable cloning vector pK18mobsacB described by Schäfer et al. (Gene 14, 69-73 (1994)). This is cleaved beforehand with the restriction enzymes EcoRI and SalI and dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim), mixed with the gluB fragment of approx. 3.7 kb, and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli Strain DH5 α (Grant et al.; Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) is then transformed with the ligation batch (Hanahan, In. DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which is supplemented with 50 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage and subsequent agarose gel electrophoresis. The plasmid is called pK18mobsacBglu2.

As described in Example 2.1, a DNA fragment which carries
the ddh gene and surrounding regions is also amplified with
the aid of the polymerase chain reaction. On the basis of
the sequence of the ddh gene cluster known for C.
glutamicum (Ishino et al., Nucleic Acids Research 15,

WO 03/040373 76

3917(1987)) (Accession Number Y00151), the following primer oligonucleotides are chosen for the PCR:

ddh_beg (SEQ ID NO: 15): 5 CTG AAT CAA AGG CGG ACA TG 3

ddh_end (SEQ ID NO: 16): 5 TCG AGC TAA ATT AGA CGT CG 3

The primers shown are synthesized by MWG Biotech and the PCR reaction is carried out by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers allow 10 amplification of a DNA fragment of approx 1.6 kb in size, which carries the ddh gene.

The amplified DNA fragment of approx. 1.6 kb in length, which the ddh gene, is identified by means of electrophoresis in a 0.8% agarose gel and isolated from the 15 gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

After purification, the fragment carrying the ddh gene is employed for ligation in the vector pK18mobsacBglu2 described. This is partly cleaved beforehand with the 20 restriction enzyme BamHI. By treatment of the vector with a Klenow polymerase (Amersham-Pharmacia, Freiburg, Germany), the overhangs of the cleaved ends are completed to blunt ends, the vector is then mixed with the DNA fragment of approx. 1.6 kb which carries the ddh gene and the mixture 25 is treated with T4 DNA ligase (Amersham-Pharmacia, Freiburg, Germany). By using Vent Polymerase (New England Biolabs, Frankfurt, Germany) for the PCR reaction, a ddhcarrying DNA fragment which has blunt ends and is suitable for ligation in the pretreated vector pK18mobsacBglu2 is 30 generated.

The E. coli strain DH50mcr (Life Technologies GmbH, Karlsruhe, Germany) is then transformed with the ligation 15

25

30

PCT/EP02/08464

batch (Hanahan, In: DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which was supplemented with 50 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage and subsequent agarose gel 10 electrophoresis. The plasmid is called pK18mobsacBglu2_1. A map of the plasmid is shown in Figure 4.

2.2 Incorporation of a second copy of the ddh gene into the chromosome (target site: gluB gene) of the strain DSM12866 by means of the replacement vector pK18mobsacBglu2_1

As described in Example 1.3, the plasmid pK18mobsacBglu2_1 described in Example 2.1 is transferred into the C. glutamicum strain DSM12866 by conjugation. Selection is 20 made for targeted recombination events in the chromosome of C. glutamicum DSM12866 as described in Example 1.3. Depending on the position of the second recombination event, after the excision the second copy of the ddh gene manifests itself in the chromosome at the gluB locus, or the original gluB locus of the host remains.

Approximately 40 to 50 colonies are tested for the phenotype "growth in the presence of sucrose" and "nongrowth in the presence of kanamycin". Approximately 20 colonies which show the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin" are investigated with the aid of the polymerase chain reaction. A DNA fragment which carries the glu region described is amplified here from the chromosomal DNA of the colonies. The same primer oligonucleotides as are described

PCT/EP02/08464 WO 03/040373 78

in Example 2.1 for the construction of the replacement plasmid are chosen for the PCR.

gluA_beg (SEQ ID NO: 13): 5 CAC GGT TGC TCA TTG TAT CC 3

gluD_end (SEQ ID NO: 14): 5' CGA GGC GAA TCA GAC TTC TT 3'

The primers allow amplification of a DNA fragment approx. 4.4 kb in size in control clones with the original glu locus. In clones with a second copy of the ddh gene in the chromosome at the gluB locus, DNA fragments with a size of approx. 6 kb are amplified.

The amplified DNA fragments are identified by means of electrophoresis in a 0.8% agarose gel.

A clone which, in addition to the copy present at the ddh locus, has a second copy of the ddh gene at the gluB locus 15 in the chromosome was identified in this manner. This clone was called strain DSM12866glu::ddh.

Example 3

10

Incorporation of a second copy of the dapA gene into the chromosome (target site: aecD gene) of the strain DSM12866 20

Construction of the replacement vector 3.1 pK18mobsacBaecD2_1

The Corynebacterium glutamicum strain ATCC13032 is used as the donor for the chromosomal DNA. From the strain ATCC13032, chromosomal DNA is isolated using the 25 conventional methods (Eikmanns et al., Microbiology 140: 1817 - 1828 (1994)). With the aid of the polymerase chain reaction, a DNA fragment which carries the aecD gene and surrounding regions is amplified. On the basis of the sequence of the aecD gene known for C. glutamicum (Rossol 30 et al., Journal of Bacteriology 174:2968-2977 (1992))

10

15

(Accession Number M89931), the following primer oligonucleotides are chosen for the PCR:

aecD_beg (SEQ ID NO: 11): 5 GAA CTT ACG CCA AGC TGT TC 3

5 aecD_end (SEQ ID NO: 12): 5` AGC ACC ACA ATC AAC GTG AG 3`

The primers shown are synthesized by MWG Biotech and the PCR reaction is carried out by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers allow amplification of a DNA fragment of approx 2.1 kb in size, which carries the aecD gene and adjacent regions.

The amplified DNA fragment of approx. 2.1 kb in length is identified by means of electrophoresis in a 0.8% agarose gel and isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

The DNA fragment purified is cleaved with the restriction enzyme BglII and EcoRV (Amersham Pharmacia, Freiburg, Germany). The ligation of the fragment in the vector pucl8 then takes place (Norrander et al., Gene 26:101-106 (1983)). This is cleaved beforehand with the restriction enzymes BamHI and SmaI and dephosphorylated, mixed with the aecD-carrying fragment of approx. 1.5 kb, and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia,

- Freiburg, Germany). The ligation batch is transformed in the E. coli strain TOP10 (Invitrogen, Leek, The Netherlands). Selection of plasmid-carrying cells is made by plating out the transformation batch on kanamycin (50 mg/l)-containing LB agar with X-Gal (64 mg/l).
- The plasmid obtained is checked by means of restriction cleavage, after isolation of the DNA, and identified in agarose gel. The resulting plasmid is called pUC18aecD.

WO 03/040373 PCT/EP02/08464

80

With the aid of the polymerase chain reaction, a further DNA fragment which carries the dapA gene and surrounding regions is amplified. On the basis of the sequence of the dapA gene known for C. glutamicum (Bonassi et al., Nucleic Acids Research 18:6421 (1990)) (Accession Number X53993 and AX127149), the following primer oligonucleotides are chosen for the PCR:

dapA_beg (SEQ ID NO: 17):
5` CGA GCC AGT GAA CAT GCA GA 3`

10 dapA_end (SEQ ID NO: 18): 5` CTT GAG CAC CTT GCG CAG CA 3`

15

20

25

The primers shown are synthesized by MWG Biotech and the PCR reaction is carried out by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers allow amplification of a DNA fragment of approx. 1.4 kb in size, which carries the dapA gene and adjacent regions.

The amplified DNA fragment of approx. 1.4 kb in length is identified by means of electrophoresis in a 0.8% agarose gel and isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

After purification, the dapA-containing DNA fragment approx. 1.4 kb in length is employed for ligation with the vector pUC18aecD described above. This is cleaved beforehand with the restriction enzyme StuI, mixed with the DNA fragment of approx. 1.4 kb, and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli strain DH50mcr (Life Technologies GmbH,
Karlsruhe, Germany) is then transformed with the ligation
batch (Hanahan, In: DNA Cloning. A Practical Approach. Vol.
ILR-Press, Cold Spring Harbor, New York, 1989).
Selection of plasmid-carrying cells is made by plating out
the transformation batch on LB agar (Sambrook et al.,

Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which was supplemented with 50 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage and subsequent agarose gel electrophoresis. The plasmid is called pUC18aecD2.

The plasmid pUC18aecD2 is cleaved with the restriction enzyme SalI and partly with EcoRI (Amersham-Pharmacia, 10 Freiburg, Germany) and after separation in an agarose gel (0.8%) with the aid of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) the fragment of approx. 2.7 kb which carries aecD and dapA is isolated from the agarose gel and employed for ligation with the mobilizable cloning 15 vector pK18mobsacB described by Schäfer et al. (Gene 14: 69-73 (1994)). This is cleaved beforehand with the restriction enzymes EcoRI and with SalI and dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim), mixed with the fragment of approx. 2.7 kb which carries aecD and dapA, and the 20 mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli strain DH5α (Grant et al.; Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) is then transformed with the ligation batch (Hanahan, In. DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which is supplemented with 50 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage and subsequent agarose gel

PCT/EP02/08464 **WO** 03/040373 **82**

electrophoresis. The plasmid is called pK18mobsacBaecD2_1. A map of the plasmid is shown in Figure 5.

Incorporation of a second copy of the dapA gene into 3.2 the chromosome (target site: aecD gene) of the strain DSM12866 by means of the replacement vector pK18mobsacBaecD2_1

As described in Example 1.3, the plasmid pk18mobsacBaecD2_1 described in Example 3.1 is transferred into the C. glutamicum strain DSM12866 by conjugation. Selection is 10 made for targeted recombination events in the chromosome of C. glutamicum DSM12866 as described in Example 1.3. Depending on the position of the second recombination event, after the excision the second copy of the dapA gene manifests itself in the chromosome at the aecD locus, or the original aecD locus of the host remains. 15

Approximately 40 to 50 colonies are tested for the phenotype "growth in the presence of sucrose" and "nongrowth in the presence of kanamycin". Approximately 20 colonies which show the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin" are investigated with the aid of the polymerase chain reaction. A DNA fragment which carries the aecD gene and surrounding regions is amplified here from the chromosomal DNA of the colonies. The same primer oligonucleotides as are described in Example 3.1 for the construction of the integration plasmid are chosen for the PCR.

aecD_beg (SEQ ID NO: 11): 5 GAA CTT ACG CCA AGC TGT TC 3

aecD_end (SEQ ID NO: 12):

5

20

25

30 5 AGC ACC ACA ATC AAC GTG AG 3

> The primers allow amplification of a DNA fragment approx. 2.1 kb in size in control clones with the original aecD locus. In clones with a second copy of the dapA gene in the

chromosome at the aecD locus, DNA fragments with a size of approx. 3.6 kb are amplified.

The amplified DNA fragments are identified by means of electrophoresis in a 0.8% agarose gel.

A clone which, in addition to the copy present at the dapA locus, has a second copy of the dapA gene at the aecD locus in the chromosome was identified in this manner. This clone was called strain DSM12866aecD::dapA.

Example 4

- 10 Incorporation of a second copy of the pyc gene in the form of the pyc allele pycP458S into the chromosome (target site: pck gene) of the strain DSM12866
 - 4.1 Construction of the replacement vector pK18mobsacBpck1_3
- The replacement vector pK18mobsacBpckl described in Example 1.5 is used as the base vector for insertion of the pyc allele.
- As described in Example 2.1, a DNA fragment which carries the pyc gene and surrounding regions is also amplified with the aid of the polymerase chain reaction. On the basis of the sequence of the pyc gene cluster known for C. glutamicum (Peters-Wendisch et al., Journal of Microbiology 144: 915-927 (1998)) (Accession Number Y09548), the following primer oligonucleotides are chosen for the PCR:
- 25 pyc_beg (SEQ ID NO: 19):
 5 TC(A CGC GT)C TTG AAG TCG TGC AGG TCA G 3`

pyc_end (SEQ ID NO: 20):
5 ` TC(A CGC GT)C GCC TCC TCC ATG AGG AAG A 3 `

The primers shown are synthesized by MWG Biotech and the 30 PCR reaction is carried out by the standard PCR method of

Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers allow amplification of a DNA fragment of approx 3.6 kb in size, which carries the pyc gene. The primers moreover contain the sequence for the cleavage site of the restriction endonuclease MluI, which is marked by parentheses in the nucleotide sequence shown above.

The amplified DNA fragment of approx. 3.6 kb in length, which carries the pyc gene, is cleaved with the restriction endonuclease MluI, identified by means of electrophoresis in a 0.8% agarose gel and isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

After purification, the fragment carrying the pyc gene is
employed for ligation in the vector pK18mobsacBpck1
described. This is cleaved beforehand with the restriction
enzyme BssHII, dephosphorylated with alkaline phosphatase
(Alkaline Phosphatase, Boehringer Mannheim, Germany), mixed
with the DNA fragment of approx. 3.6 kb which carries the
pyc gene, and the mixture is treated with T4 DNA Ligase
(Amersham-Pharmacia, Freiburg, Germany).

The E. coli strain DH50mcr (Life Technologies GmbH, Karlsruhe, Germany) is then transformed with the ligation batch (Hanahan, In: DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which was supplemented with 50 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage and subsequent agarose gel electrophoresis. The plasmid is called pK18mobsacBpck1_2.

4.2 Construction of the pyc allele pyc P458S by means of site-specific mutagenesis of the wild-type pyc gene

The site-directed mutagenesis is carried out with the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA). EP-A-1108790 describes a point mutation in the pyc gene for C. glutamicum which allows improved L-lysine production. On the basis of the point mutation in the nucleotide sequence of cytosine to thymine in the pyc gene at position 1372, replacement in the amino acid sequence derived therefrom of proline for serine at position 458 results. The allele is called pyc P458S. To generate the mutation described, the following primer oligonucleotides are chosen for the linear amplification:

P458S-1 (SEQ ID NO: 21):

20

25

30

15 5' GGATTCATTGCCGATCAC (TCG) CACCTCCTTCAGGCTCCA 3'

P458S-2 (SEQ ID NO: 22):

5'GTGGAGGAAGTCCGAGGT (CGA) GTGATCGGCAATGAATCC 3'

The primers shown are synthesized by MWG Biotech. The codon for serine, which is to replace the proline at position 458, is marked by parentheses in the nucleotide sequence shown above. The plasmid pK18mobsacBpck1_2 described in Example 4.1 is employed with the two primers, which are each complementary to a strand of the plasmid, for linear amplification by means of Pfu Turbo DNA polymerase. By this lengthening of the primers, a mutated plasmid with broken circular strands is formed. The product of the linear amplification is treated with DpnI - this endonuclease cleaves the methylated and half-methylated template DNA specifically. The newly synthesized broken, mutated vector DNA is transformed in the E. coli strain XL1 Blue (Bullock, Fernandez and Short, BioTechniques (5) 376-379 (1987)). After the transformation, the XL1 Blue cells repair the breaks in the mutated plasmids. Selection of the transformants was carried out on LB medium with kanamycin

5

25

30

50 mg/l. The plasmid obtained is checked by means of restriction cleavage, after isolation of the DNA, and identified in agarose gel. The DNA sequence of the mutated DNA fragment is checked by sequencing. The sequence of the PCR product coincides with the sequence described Ohnishi et al. (2002). The resulting plasmid is called pK18mobsacBpck1_3. A map of the plasmid is shown in Figure 6.

Incorporation of a second copy of the pyc gene in the form of the pyc allele pycP458S into the chromosome (target site pck gene) of the strain DSM12866 by means of the replacement vector pk18mobsacBpck1_3

The plasmid pK18mobsacBpck1_3 described in Example 4.2 is transferred as described in Example 1.3 into the C.

15 glutamicum strain DSM12866 by conjugation. Selection is made for targeted recombination events in the chromosome of C. glutamicum DSM12866 as described in Example 1.3.

Depending on the position of the second recombination event, after the excision the second copy of the pyc allele manifests itself in the chromosome at the pck locus, or the original pck locus of the host remains.

Approximately 40 to 50 colonies are tested for the phenotype "growth in the presence of sucrose" and "nongrowth in the presence of kanamycin". Approximately 20 colonies which show the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin" are investigated with the aid of the polymerase chain reaction. A DNA fragment which carries the pck gene and surrounding regions is amplified here from the chromosomal DNA of the colonies. The same primer oligonucleotides as are described in Example 1.5 for the construction of the replacement plasmid are chosen for the PCR.

pck_beg (SEQ ID NO: 9):
5 TA(A GAT CT) G CCG GCA TGA CTT CAG TTT 3)

PCT/EP02/08464

pck_end (SEQ ID NO: 10): 5 AC (A GAT CT) G GTG GGA GCC TTT CTT GTT ATT3

The primers allow amplification of a DNA fragment approx. 2.9 kb in size in control clones with the original pck locus. In clones with a second copy of the pyc allele in the chromosome at the pck locus, DNA fragments with a size of approx. 6.5 kb are amplified.

The amplified DNA fragments are identified by means of electrophoresis in a 0.8% agarose gel.

A clone which, in addition to the copy of the wild-type 10 gene present at the pyc locus, has a second copy of the pyc gene in the form of the pyc allele pycP458S at the pck locus in the chromosome was identified in this manner. This clone was called strain DSM12866pck::pyc.

15 Example 5

Preparation of Lysine

The C. glutamicum strains DSM13994glu::lysC, DSM12866glu::lysC, DSM12866pck::lysC, DSM12866aecD::lysC, DSM12866glu::ddh, DSM12866aecD::dapA and DSM12866pck::pyc obtained in Example 1, 2, 3 and 4 are cultured in a 20 nutrient medium suitable for the production of lysine and the lysine content in the culture supernatant was determined.

For this, the cultures are first incubated on a brain-heart agar plate (Merck, Darmstadt, Germany) for 24 hours at 25 33°C. Starting from this agar plate culture, a preculture is seeded (10 ml medium in a 100 ml conical flask). The medium MM is used as the medium for the preculture. The preculture is incubated for 24 hours at 33°C at 240 rpm on a shaking machine. A main culture is seeded from this 30 preculture such that the initial OD (660 nm) of the main

culture is 0.1 OD. The Medium MM is also used for the main culture.

Medium MM

CSL	5 g/l
MOPS	20 g/l .
Glucose (autoclaved separately)	50 g/l
Salts:	
(NH ₄) ₂ SO ₄	25 g/l
KH_2PO_4	0.1 g/l
$MgSO_4 * 7 H_2O$	1.0 g/l
CaCl ₂ * 2 H ₂ O	10 mg/l
FeSO ₄ * 7 H ₂ O	10 mg/l
$MnSO_4 * H_2O$	5.0 mg/l
Biotin (sterile-filtered)	0.3 mg/1
Thiamine * HCl (sterile-filtered)	0.2 mg/l
CaCO ₃	25 g/l

The CSL (corn steep liquor), MOPS
(morpholinopropanesulfonic acid) and the salt solution are brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions, as well as the CaCO3 autoclaved in the dry state, are then added.

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Culturing is carried out at 33°C and 80% atmospheric humidity.

After 48 hours, the OD is determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of lysine formed is determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivation with ninhydrin detection.

The result of the experiment is shown in Table 10.

Table 10

Strain	OD (660 nm)	Lysine HCl
DSM13994	12.0	g/l 19.1
DSMIJJJ4	12.0	19.1
DSM13994glu::lysC	9.9	20.0
DSM12866	12.5	14.9
DSM15039	11.4	16.2
DSM12866pck::lysC	12.6	16.5
DSM12866aecD::lysC	12.0	15.9
DSM12866glu::ddh	11.0	15.5
DSM12866aecD::dapA	11.1	16.2
DSM12866pck::pyc	10.9	16.9

Brief Description of the Figures:

The base pair numbers stated are approximate values obtained in the context of reproducibility of measurements.

Figure 1: Map of the plasmid pK18mobsacBglu1_1.

The abbreviations and designations used have the following meaning:

KanR: Kanamycin resistance gene

HindIII: Cleavage site of the restriction enzyme

HindIII

BamHI: Cleavage site of the restriction enzyme

BamHI

lysC: lysC^{FBR} allele, lysC T311I

'gluA: 3' terminal fragment of the gluA gene

gluB': 5' terminal fragment of the gluB gene

'gluB: 3' terminal fragment of the gluB gene

gluC': 5' terminal fragment of the gluC gene

sacB: sacB gene

RP4mob: mob region with the replication origin for

the transfer (oriT)

oriV: Replication origin V

Figure 2: Map of the plasmid pK18mobsacBaecD1_1.

The abbreviations and designations used have the following meaning:

Kanamycin resistance gene

SalI: Cleavage site of the restriction enzyme SalI

lysC: lysC^{FBR} allele, lysC T311I

aecD': 5' terminal fragment of the aecD gene

'aecD: 3' terminal fragment of the aecD gene

sacB: sacB gene

RP4mob: mob region with the replication origin for

the transfer (oriT)

oriV: Replication origin V

Figure 3: Map of the plasmid pK18mobsacBpck1_1.

The abbreviations and designations used have the following meaning:

KanR: Kanamycin resistance gene

BamHI: Cleavage site of the restriction enzyme

BamHI

lysC: lysC^{FBR} allele, lysC T311I

pck': 5' terminal fragment of the pck gene

'pck: 3' terminal fragment of the pck gene

sacB: sacB gene

RP4mob: mob region with the replication origin for

the transfer (oriT)

oriV: Replication origin V

Figure 4: Map of the plasmid pK18mobsacBgluB2_1.

The abbreviations and designations used have the following meaning:

KanR: Kanamycin resistance gene

SalI Cleavage site of the restriction enzyme SalI

EcoRI Cleavage site of the restriction enzyme

EcoRI

BamHI: Cleavage site of the restriction enzyme

BamHI

ddh: ddh gene

gluA gene

gluB': 5' terminal fragment of the gluB gene

'gluB: 3' terminal fragment of the gluB gene

gluC gene

gluD': 5' terminal fragment of the gluD gene

sacB: sacB gene

RP4mob: mob region with the replication origin for

the transfer (oriT)

oriV: Replication origin V

Figure 5: Map of the plasmid pK18mobsacBaecD2_1.

The abbreviations and designations used have the following meaning:

Kanamycin resistance gene

EcoRI Cleavage site of the restriction enzyme

ECORI

SalI: Cleavage site of the restriction enzyme SalI

dapA: dapA gene

aecD': 5' terminal fragment of the aecD gene

'aecD: 3' terminal fragment of the aecD gene

sacB: sacB gene

RP4mob: mob region with the replication origin for

the transfer (oriT)

oriV: Replication origin V

Figure 6: Map of the plasmid pK18mobsacBpck1_3.

The abbreviations and designations used have the following meaning:

KanR: Kanamycin resistance gene

pyc: pyc allele, pyc P458S

pck': 5' terminal fragment of the pck gene

'pck: 3' terminal fragment of the pck gene

sacB: sacB gene

RP4mob: mob region with the replication origin for

the transfer (oriT)

oriV: Replication origin V

PCT/EP02/08464 DSIMZ Deutsche Sammlung von Mikroorganismen und Zelikulturen GmbH

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa AG

Kantstr. 2

33790 Halle (Westf.)

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM			
	in reference given by the DEPOSITOR: M12866glu::lysC	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 15039	
II. SCIENT	IFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIG	NATION	
The microor	The microorganism identified under I. above was accompanied by:		
(Mark with	 (x) a scientific description (x) a proposed taxonomic designation a cross where applicable). 		
III. RECEIP	T AND ACCEPTANCE		
This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2002-06-05 (Date of the original deposit).			
IV. RECEIP	T OF REQUEST FOR CONVERSION		
The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on for conversion). (date of original deposit) (date of receipt of request			
V. INTERN	ATIONAL DEPOSITARY AUTHORITY		
Name: Address:	DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 2002-06-06	

Where Rule 6.4 (d) applies, such date is the date on which the status of international depository authority was acquired. Form DSMZ-BP/4 (sole-page) 12/2001

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE



INTERNATIONAL FORM

Degussa AG

Kantstr. 2

33790 Halle (Westf.)

VIABILITY STATEMENT issued pursuant to Rule 10.2 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

L DEPOSIT	OR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Address:	Degussa AG Kantstr. 2 33790 Halle (Westf.)	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 15039 Date of the deposit or the transfer!: 2002-06-05
III. VIABILI	ITY STATEMENT	
On that date (χ)	y of the microorganism identified under II above was tested on the said microorganism was y viable o longer viable	2002-06-05
IV. CONDI	TIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PE	RFORMED ⁴
V. INTERN	ATIONAL DEPOSITARY AUTHORITY	
Name: Address:	DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.

Mark with a cross the applicable box.

Fill in if the information has been requested and if the results of the test were negative.

Form DSMZ-BP/9 (sole page) 12/2001

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE



INTERNATIONAL FORM

Degussa AG

Kantstr. 2

33790 Halle (Westf.)

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM		
Identification reference given by the DEPOSITOR: DH5alphamcr/pK18mobsacBaecD1_	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 15040	
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED T	AXONOMIC DESIGNATION	
The microorganism identified under I. above was accompa	nied by:	
(x) a scientific description (x) a proposed taxonomic designation (Mark with a cross where applicable).		
IIL RECEIPT AND ACCEPTANCE		
This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2002-06-05 (Date of the original deposit).		
IV. RECEIPT OF REQUEST FOR CONVERSION		
The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on for conversion). (date of original deposit) (date of receipt of request		
V. INTERNATIONAL DEPOSITARY AUTHORITY		
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTI Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 2002-06-06	

Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired. Form DSMZ-BP/4 (sole page) 12/2001

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE



INTERNATIONAL FORM

Degussa AG

Kantstr. 2

33790 Halle (Westf.)

VIABILITY STATEMENT issued pursuant to Rule 10.2 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

. DEPOSIT	OR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Address:	Degussa AG Kantstr. 2 33790 Halle (Westf.)	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 15040 Date of the deposit or the transfer!: 2002-06-05
II. VLABILI	TTY STATEMENT	
On that date (X)	y of the microorganism identified under II above was tested on the said microorganism was y viable no longer viable	2002-06-05
IV. CONDIT	TIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PE	RFORMED ⁴
V. INTERN	ATIONAL DEPOSITARY AUTHORITY	
Name:	DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Mascheroder Weg 1b	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
Mark with a cross the applicable box.
Fill in if the information has been requested and if the results of the test were negative.

Form DSMZ-BP/9 (sole page) 12/2001

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa AG Kantstr. 2 33790 Halle/Künsebeck

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM		
Identification reference given by the DEPOSITOR: DH5alphamcr/ pK18mobsacBglu1_1	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 14243	
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DES	IGNATION	
The microorganism identified under I. above was accompanied by:		
(X) a scientific description (X) a proposed taxonomic designation (Mark with a cross where applicable).		
III. RECEIPT AND ACCEPTANCE		
This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2001-04-20 (Date of the original deposit) ¹ .		
IV. RECEIPT OF REQUEST FOR CONVERSION		
The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).		
V. INTERNATIONAL DEPOSITARY AUTHORITY		
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):	
	Date: 2001-04-26	

Form DSMZ-BP/4 (sole page) 0196

Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

BUDAPEST TREATY ON THE INTERNATIONAL ECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa AG Kantstr. 2 33790 Halle/Künsebeck

> VIABILITY STATEMENT issued pursuant to Rule 10.2 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Degussa AG Kantstr. 2 Address: 33790 Halle/Künsebeck	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 14243 Date of the deposit or the transfer ¹ : 2001-04-20
II. VIABILITY STATEMENT	· · · · · · · · · · · · · · · · · · ·
The viability of the microorganism identified under II above was tested on On that date, the said microorganism was (X) vlable () no longer viable IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of anthorized official(s): Date: 2001-04-26

Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test. Mark with a cross the applicable box.

Fill in if the information has been requested and if the results of the test were negative.

What is claimed is:

- Coryneform bacteria which produce chemical compounds, wherein these have, in addition to at least one copy, present at the natural site (locus), of an open reading 5 frame (ORF), gene or allele which codes for the synthesis of a protein or an RNA, a second, optionally third or fourth copy of the open reading frame (ORF), gene or allele in question at a second, optionally third or fourth site in a form integrated into the 10 chromosome, no nucleotide sequence which is capable of/enables episomal replication or transposition in microorganisms and no nucleotide sequence(s) which impart(s) resistance to antibiotics being present at the second, optionally third or fourth site, and the 15 second, optionally third or fourth site not relating to open reading frames (ORF), genes or alleles which are essential for the growth of the bacteria and the production of the desired compound.
- 2. Coryneform bacteria according to claim 1 which produce chemical compounds, wherein the coryneform bacteria belong to the genus Corynebacterium.
 - 3. Coryneform bacteria of the genus Corynebacterium according to claim 2 which produce chemical compounds, wherein these belong to the species Corynebacterium glutamicum.
 - 4. Coryneform bacteria according to claim 1 which produce chemical compounds, wherein the chemical compound is a compound chosen from the group consisting of L-amino acids, vitamins, nucleosides and nucleotides.
- 5. Coryneform bacteria according to claim 1 which produce chemical compounds, wherein the chemical compound is one or more L-amino acids chosen from the group consisting of L-aspartic acid, L-asparagine, L-

threonine, L-serine, L-glutamic acid, L-glutamine, glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan, L-proline and L-arginine.

6. Coryneform bacteria according to claims 1 and 4 which produce chemical compounds, wherein the L-amino acid is L-lysine, and these bacteria have, in addition to at least one copy of an open reading frame (ORF), gene or allele of lysine production present at the natural site (locus), in each case a second, optionally third or fourth copy of the open reading frame (ORF), gene or allele of lysine production in question at in each case a second, optionally third or fourth site in a form integrated into the chromosome.

- 7. Coryneform bacteria according to claim 6 which produce L-lysine, wherein the coryneform bacteria belong to the genus Corynebacterium.
- 8. Coryneform bacteria of the genus Corynebacterium

 20 according to claim 7 which produce L-lysine, wherein these belong to the species Corynebacterium glutamicum.
- 9. Coryneform bacteria according to claim 6 which produce L-lysine, wherein the open reading frame (ORF), gene or allele of lysine production is one or more open reading frame(s), one or more gene(s) or allele(s) chosen from the group consisting of accBC, accDA, cstA, cysD, cysE, cysH, cysK, cysN, cysQ, dapA, dapB, dapC, dapD, dapE, dapF, ddh, dps, eno, gap, gap2, gdh, gnd, lysC, lysCFBR, lysE, msiK, opcA, oxyR, ppc, ppcFBR, pgk, pknA, pknB, pknD, pknG, ppsA, ptsH, ptsI, ptsM, pyc, pyc P458S, sigC, sigD, sigE, sigH, sigM, tal, thyA, tkt, tpi, zwa1, zwf and zwf A213T.

10. Coryneform bacteria according to claim 6 which produce L-lysine, wherein the open reading frame, gene or allele of lysine production is one or more gene(s) or allele(s) chosen from the group consisting of dapA, ddh, lysCFBR and pyc P458S.

5

- 11. Coryneform bacteria according to claim 6 which produce L-lysine, wherein the open reading frame, gene or allele of lysine production is a lysCFBR allele which codes for a feed back resistant form of aspartate kinase.
- Coryneform bacteria according to claim 11 which produce L-lysine, wherein the feed back resistant form of aspartate kinase coded by the lysCFBR allele contains an amino acid sequence according to SEQ ID NO:2, SEQ ID NO:2 containing one or more amino acid replacements chosen from the group consisting of A279T, A279V, S301F, T308I, S301Y, G345D, R320G, T311I and S381F.
 - 13. Coryneform bacteria according to claim 11 which produce L-lysine, wherein the feed back resistant form of aspartate kinase coded by the lysCFBR allele includes an amino acid sequence according to SEQ ID NO:4.
 - 14. Coryneform bacteria according to claim 11 which produce L-lysine, wherein the coding region of the lysC^{FBR} allele includes the nucleotide sequence of SEQ ID NO:3.
- 25 15. Coryneform bacteria according to claim 6 which produce L-lysine, wherein the particular second, optionally third or fourth site is a gene chosen from the group consisting of aecD, ccpAl, ccpA2, citA, citB, citE, fda, gluA, gluB, gluC, gluD, luxR, luxS, lysR1, lysR2, lysR3, menE, mqo, pck, pgi and poxB.
 - 16. Coryneform bacteria according to claim 6 which produce L-lysine, wherein the particular second, optionally third or fourth site is a site chosen from the group

WO 03/040373 PCT/EP02/08464

consisting of intergenic regions of the chromosome, prophages contained in the chromosome and defective phages contained in the chromosome.

- 17. Coryneform bacteria according to claim 15 which produce L-lysine, wherein the particular second, optionally third or fourth site is the aecD gene site.
 - 18. Coryneform bacteria according to claim 15 which produce L-lysine, wherein the particular second, optionally third or fourth site is the gluB gene site.
- 10 19. Coryneform bacteria according to claim 15 which produce L-lysine, wherein the particular second, optionally third or fourth site is the pck gene site.
 - 20. Process for the preparation of chemical compounds by fermentation of coryneform bacteria, in which the following steps are carried out:

- a) fermentation of coryneform bacteria, which
- which have, in addition to at least one copy, a1) present at the natural site (locus), of an open reading frame (ORF), gene or allele which codes 20 for the synthesis of a protein or an RNA, a second, optionally third or fourth copy of this open reading frame (ORF), gene or allele at a second, optionally third or fourth site in a form integrated into the chromosome, no nucleotide 25 sequence which is capable of/enables episomal replication or transposition in microorganisms and no nucleotide sequence(s) which impart(s) resistance to antibiotics being present at the second, optionally third or fourth site, and the 30 second, optionally third or fourth site not relating to open reading frames (ORF), genes or alleles which are essential for the growth of the

5

PCT/EP02/08464 **WO** 03/040373

> bacteria and the production of the desired compound, and

- in which the intracellular activity of the a2) corresponding protein is increased, in particular the nucleotide sequence which codes for this protein is over-expressed,
 - concentration of the chemical compound(s) in the C) fermentation broth and/or in the cells of the bacteria,
- isolation of the chemical compound(s), optionally 10 d)
 - with constituents from the fermentation broth e) and/or the biomass to the extent of > (greater than) 0 to 100 wt.%.
- 21. Process according to claim 20, wherein the coryneform bacteria belong to the genus Corynebacterium. 15
 - 22. Process according to claim 20, wherein the coryneform bacteria of the genus Corynebacterium belong to the species Corynebacterium glutamicum.
- 23. Process according to claim 20, wherein the chemical compound is a compound chosen from the group consisting 20 of L-amino acids, vitamins, nucleosides and nucleotides.
- 24. Process according to claim 20, wherein the chemical compound is one or more L-amino acids chosen from the group consisting of L-aspartic acid, L-asparagine, L-25 threonine, L-serine, L-glutamic acid, L-glutamine, glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan, L-proline and Larginine. 30

25. Process according to claim 24, wherein the chemical compound is L-lysine.

- 26. Process for the preparation of L-lysine, which comprises the following steps:
- a) fermentation of coryneform bacteria which have, in addition to at least one copy of an open reading frame (ORF), gene or allele of lysine production present at the natural site (locus), in each case a second, optionally third or fourth copy of the open reading frame (ORF), gene or allele of lysine production in question at in each case a second, optionally third or fourth site in a form integrated into the chromosome
- under conditions which allow expression of the said open reading frames (ORF), genes or alleles mentioned.
- 27. Process for the preparation of L-lysine according to claim 26, wherein the open reading frame (ORF), gene or allele of lysine production is an open reading frame, a gene or allele chosen from the group consisting of accBC, accDA, cstA, cysD, cysE, cysH, cysK, cysN, cysQ, dapA, dapB, dapC, dapD, dapE, dapF, ddh, dps, eno, gap, gap2, gdh, gnd, lysC, lysCFBR, lysE, msiK, opcA, oxyR, ppc, ppcFBR, pgk, pknA, pknB, pknD, pknG, ppsA, ptsH, ptsI, ptsM, pyc, pyc P458S, sigC, sigD, sigE, sigH, sigM, tal, thyA, tkt, tpi, zwa1, zwf and zwf A213T.
- 28. Process for the preparation of L-lysine according to claim 26, wherein the open reading frame (ORF), gene or allele of lysine production is a gene or allele chosen from the group consisting of dapA, ddh, lysCFBR and pyc P458S.

29. Process for the preparation of L-lysine according to claim 26, wherein the open reading frame (ORF), gene or allele of lysine production is a lysCFBR allele which codes for a feed back resistant form of aspartate kinase.

5

10

- 30. Process for the preparation of L-lysine according to claim 29, wherein the feed back resistant form of aspartate kinase coded by the lysCFBR allele contains an amino acid sequence according to SEQ ID NO:2, SEQ ID NO:2 containing one or more amino acid replacements chosen from the group consisting of A279T, A279V, S301F, T308I, S301Y, G345D, R320G, T311I and S381F.
- 31. Process for the preparation of L-lysine according to claim 29, wherein the feed back resistant form of aspartate kinase coded by the lysCFBR allele includes an amino acid sequence according to SEQ ID NO:4.
- 32. Process for the preparation of L-lysine according to claim 29, wherein the coding region of the lysC^{FBR} allele includes the nucleotide sequence of SEQ ID NO:3.
- 20 33. Process for the preparation of L-lysine according to claim 26, wherein the particular second, optionally third or fourth site is a site chosen from the group consisting of aecD, ccpAl, ccpA2, citA, citB, citE, fda, gluA, gluB, gluC, gluD, luxR, luxS, lysR1, lysR2, lysR3, menE, mqo, pck, pgi and poxB.
 - 34. Process for the preparation of L-lysine according to claim 26, wherein the second, optionally third or fourth site is the aecD gene site.
- 35. Process for the preparation of L-lysine according to claim 26, wherein the second, optionally third or fourth site is the gluB gene site.

- 36. Process for the preparation of L-lysine according to claim 26, wherein the second, optionally third or fourth site is the pck gene site.
- 37. Process for the production of coryneform bacteria which produce one or more chemical compounds, which comprises
 - a) isolating the nucleotide sequence of at least one desired ORF, gene or allele which codes for a protein or an RNA, optionally including the expression and/or regulation signals, preferably from coryneform bacteria,

10

15

- b) providing the 5' and the 3' end of the ORF, gene or allele with nucleotide sequences of the target site,
- c) preferably incorporating the nucleotide sequence of the desired ORF, gene or allele provided with nucleotide sequences of the target site into a vector which does not replicate or replicates to only a limited extent in coryneform bacteria,
- d) transferring the nucleotide sequences according to b) or c) into coryneform bacteria, and
- e) isolating coryneform bacteria in which the nucleotide sequence(s) according to a) is incorporated at the target site, no nucleotide sequence(s) which is(are) capable of/enable(s) episomal replication or transposition in microorganisms, and no nucleotide sequence(s) which impart(s) resistance to antibiotics remaining at the target site.
- 38. Plasmid pK18mobsacBglu1_1 shown in Figure 1 and deposited in the form of a pure culture of the strain
 E. coli DH5cmcr/pK18mobsacBglu1_1 (= DH5alpha mcr/pK18mobsacBglu1_1) under number DSM14243.

- 39. Plasmid pK18mobsacBaecD1_1 shown in Figure 2 and deposited in the form of a pure culture of the strain E. coli DH5cmcr/pK18mobsacBaecD1_1 (= DH5alphamcr/pK18mobsacBaecD1_1) under number DSM15040.
- 5 40. Corynebacterium glutamicum strain DSM12866glu::lysC deposited in the form of a pure culture under number DSM15039.

Figure 1: Plasmid pK18mobsacBglu1_1

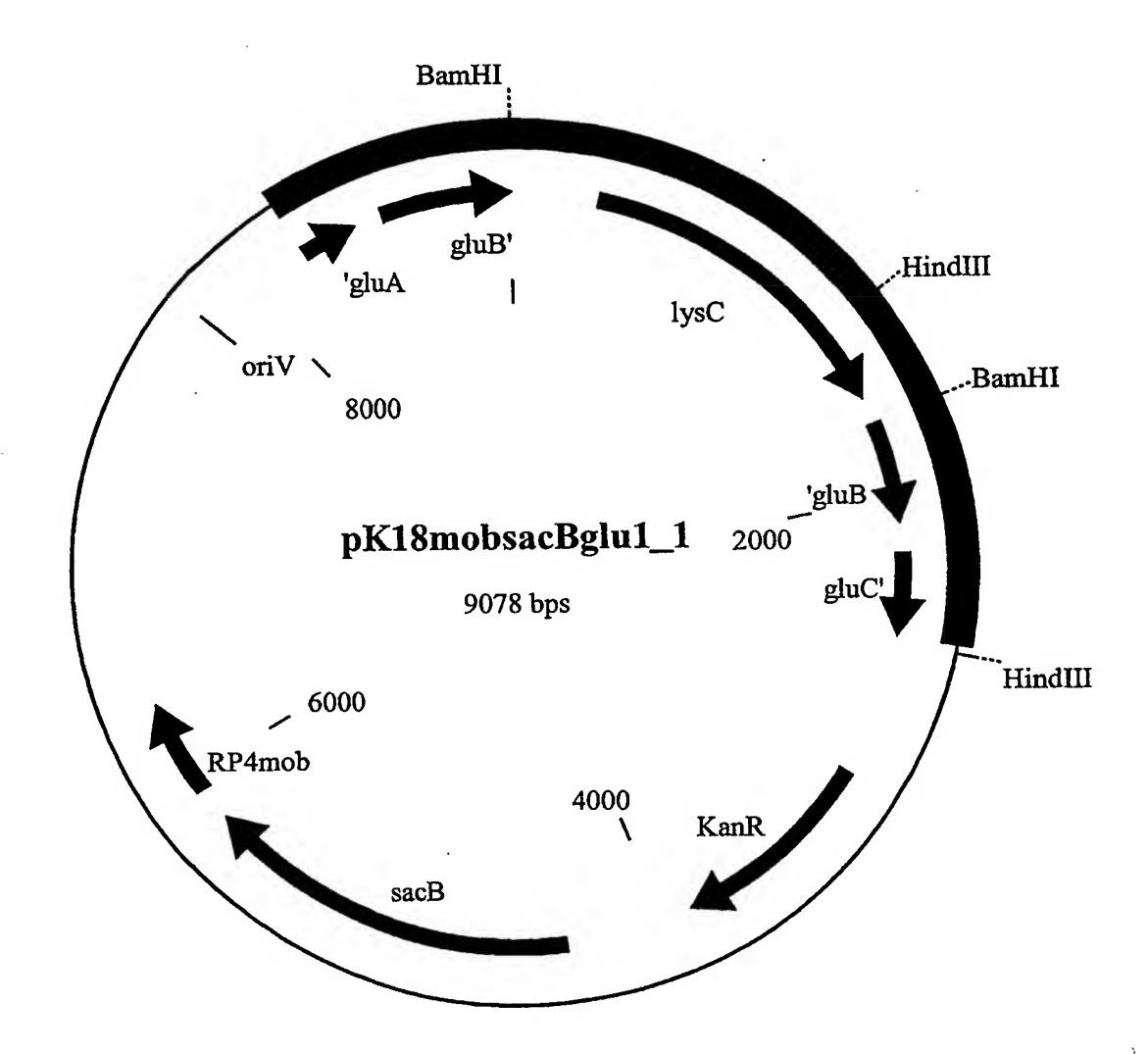


Figure 2: Plasmid pK18mobsacBaecD1_1

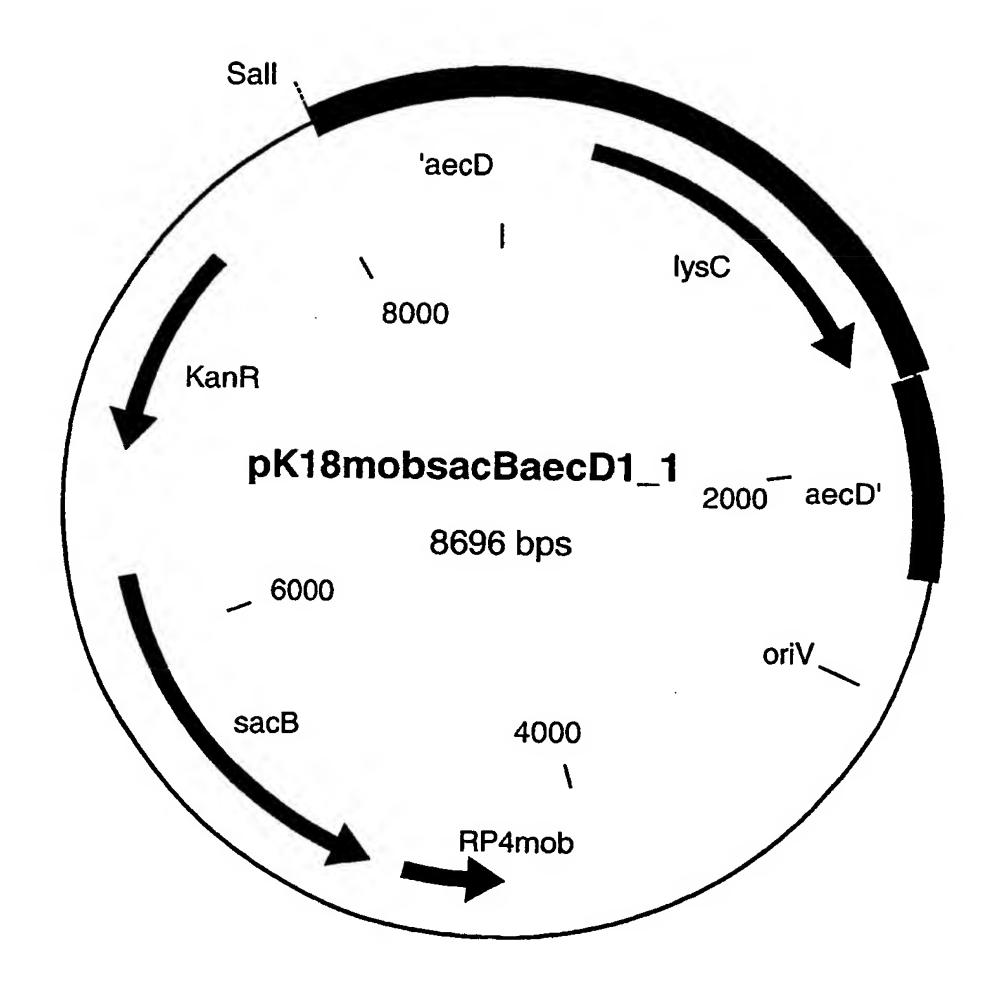


Figure 3: Plasmid pK18mobsacBpck1_1

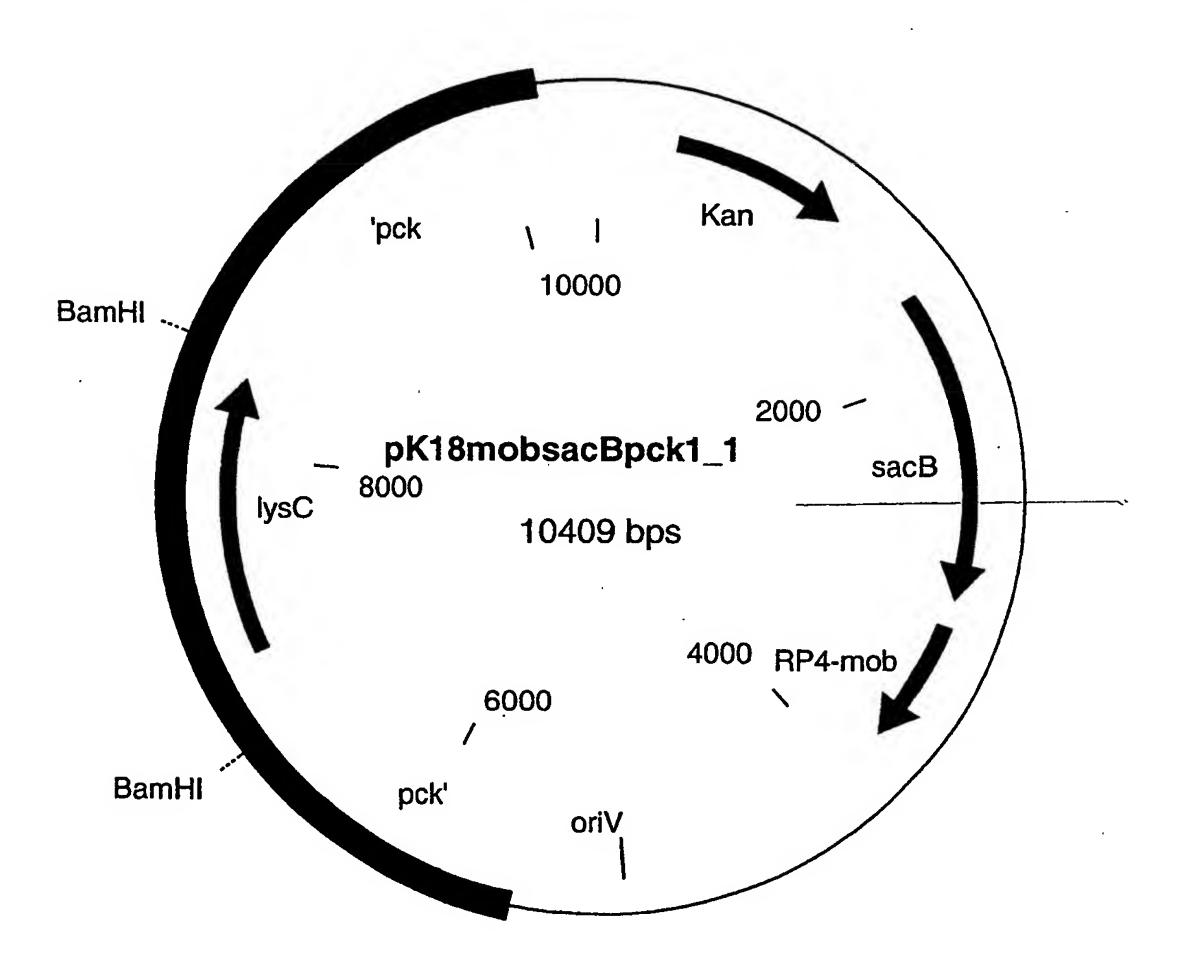


Figure 4: Plasmid pK18mobsacBgluB2_1

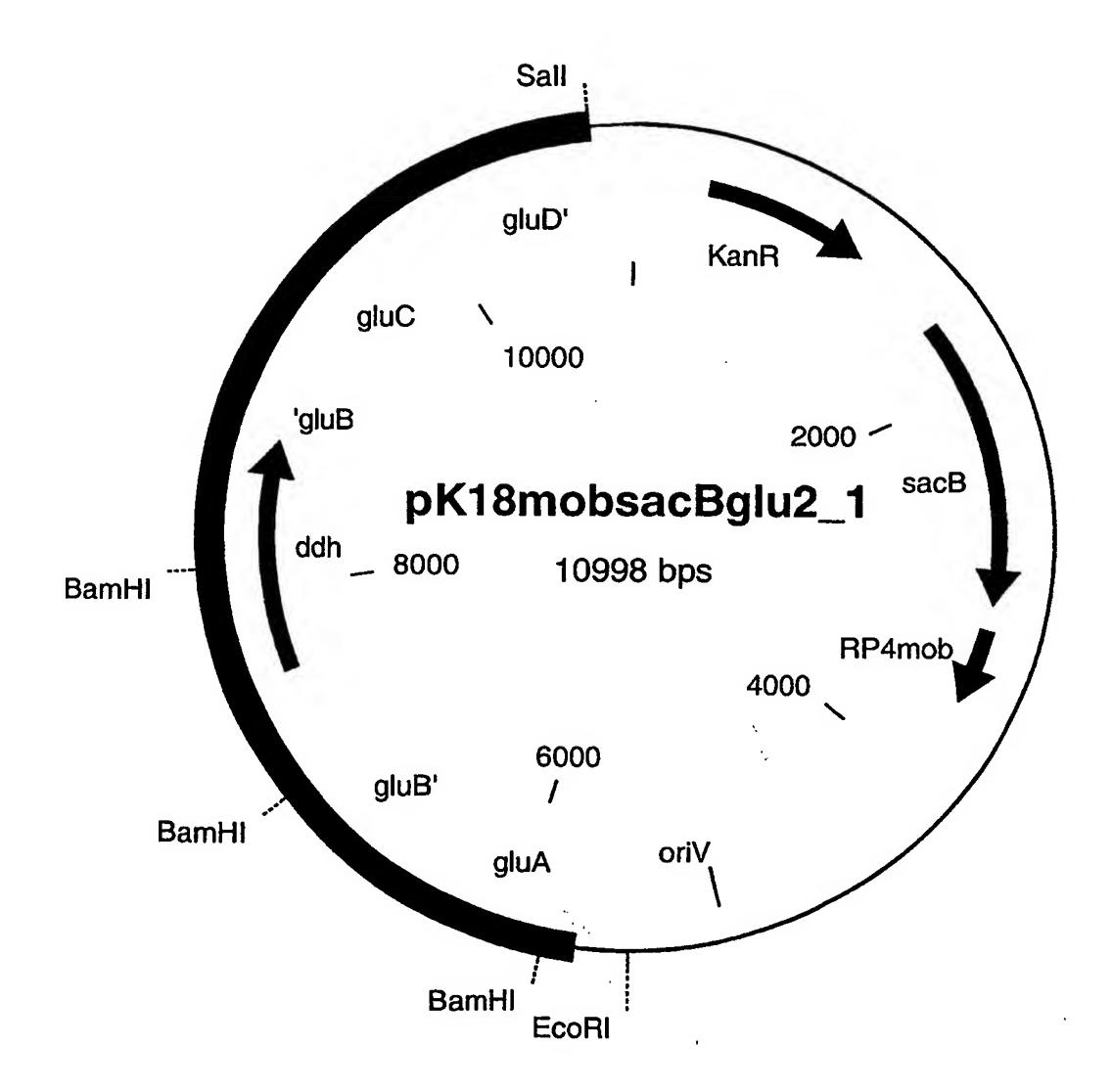


Figure 5: Plasmid pK18mobsacBaecD2_1

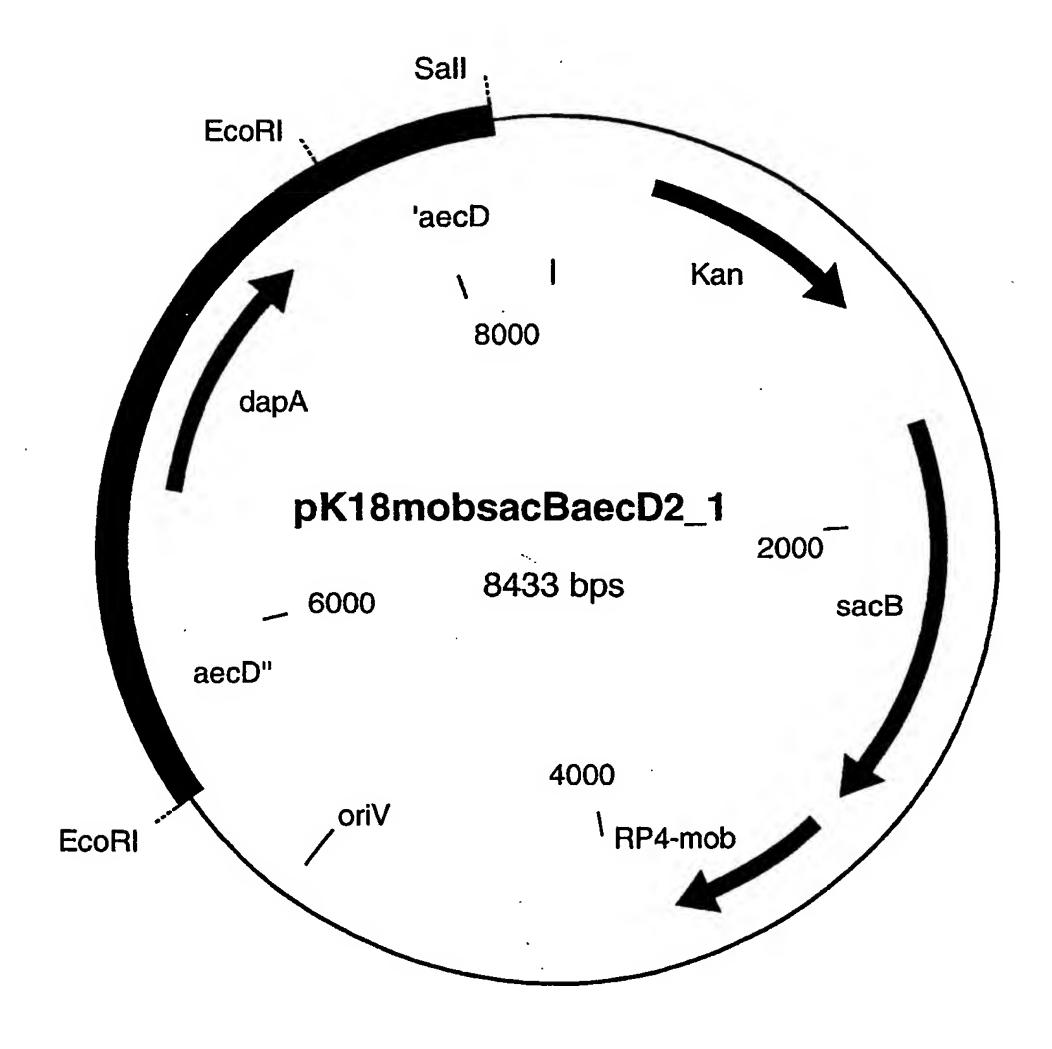
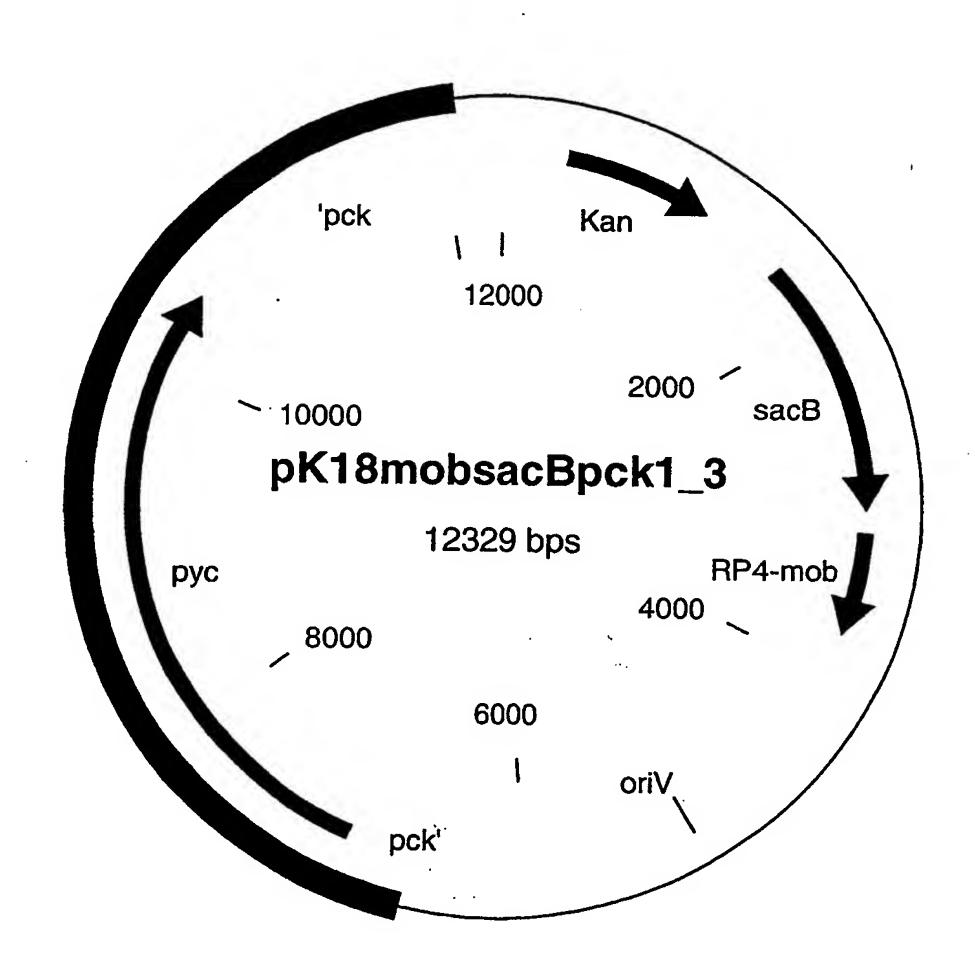


Figure 6: Plasmid pK18mobsacBpck1_3



SEQUENCE LISTING

5	<110>	De	guss	a AG	3												
	<120>	Co	ryne	forn	n bac	cteri	ia wh	nich	prod	duce	che	mica:	l co	mpou	nds	I	
10	<130>	01	.0301	BT													
	<160>	22	2														
	<170>	Pá	atent	:In v	vers	ion :	3.1										
15	<210><211><212><213>	12 Di	AV	ebac	teri	um gi	luta	micu	m								
20	<220><221><222><223>	C1	1)	* _	•	e ge	ne										
25	<400>	. 1															
20	gtg g Met A	icc (ctg	Val	gta Val 5	cag Gln	aaa Lys	tat Tyr	ggc Gly	ggt Gly 10	tcc Ser	tcg Ser	ctt Leu	gag Glu	agt Ser 15	gcg Ala	48
30	gaa c Glu A	agc Arg	Ile	aga Arg 20	aac Asn	gtc Val	gct Ala	gaa Glu	cgg Arg 25	atc Ile	gtt Val	gcc Ala	acc Thr	aag Lys 30	aag Lys	gct Ala	96
35	gga a Gly A	Asn	gat Asp 35	gtc Val	gtg Val	gtt Val	gtc Val	tgc Cys 40	tcc Ser	gca Ala	atg Met	gga Gly	gac Asp 45	acc Thr	acg Thr	gat Asp	144
40	gaa d Glu I	ctt Leu 50	cta Leu	gaa Glu	ctt Leu	gca Ala	gcg Ala 55	gca Ala	gtg Val	aat Asn	ccc Pro	gtt Val 60	ccg Pro	cca Pro	gct Ala	cgt Arg	192
45	gaa a Glu 1 65	atg Met	gat Asp	atg Met	ctc Leu	ctg Leu 70	act Thr	gct Ala	ggt Gly	gag Glu	cgt Arg 75	att Ile	tct Ser	aac Asn	gct Ala	ctc Leu 80	240
5 0	gtc (gcc Ala	atg Met	gct Ala	att Ile 85	gag Glu	tcc Ser	ctt Leu	ggc	gca Ala 90	gaa Glu	gcc Ala	caa Gln	tct Ser	ttc Phe 95	acg Thr	288
50	ggc Gly	tct Ser	cag Gln	gct Ala 100	ggt Gly	gtg Val	ctc Leu	acc Thr	acc Thr 105	gag Glu	cgc Arg	cac His	gga Gly	aac Asn 110	gca Ala	cgc Arg	336
55	att Ile	gtt Val	gat Asp 115	Val	act Thr	cca Pro	ggt Gly	cgt Arg 120	Val	cgt Arg	gaa Glu	gca Ala	ctc Leu 125	gat Asp	gag Glu	ggc Gly	384
60	Lys	atc Ile 130	Cys	att Ile	gtt Val	gct Ala	ggt Gly 135	Phe	cạg Gln	ggt Gly	gtt Val	aat Asn 140	aaa Lys	gaa Glu	acc Thr	cgc Arg	432

PCT/EP02/08464

	gat Asp 145	gtc Val	acc Thr	acg Thr	ttg Leu	ggt Gly 150	cgt Arg	ggt Gly	ggt Gly	tct Ser	gac Asp 155	Thr	act Thr	gca Ala	gtt Val	gcg Ala 160	480
5	ttg Leu	gca Ala	gct Ala	gct Ala	ttg Leu 165	aac Asn	gct Ala	·gat Asp	gtg Val	tgt Cys 170	gag Glu	att Ile	tac Tyr	tcg Ser	gac Asp 175		528
10	gac Asp	ggt Gly	gtg Val	tat Tyr 180	acc Thr	gct Ala	gac Asp	ccg Pro	cgc Arg 185	Ile	gtt Val	cct Pro	aat Asn	gca Ala 190	Gln	aag Lys	576
15	ctg Leu	gaa Glu	aag Lys 195	ctc Leu	agc Ser	ttc Phe	gaa Glu	gaa Glu 200	atg Met	ctg Leu	gaa Glu	ctt Leu	gct Ala 205	Ala	gtt Val	ggc Gly	624
20	tcc Ser	aag Lys 210	att Ile	ttg Leu	gtg Val	ctg Leu	cgc Arg 215	agt Ser	gtt Val	gaa Glu	tac Tyr	gct Ala 220	Arg	gca Ala	ttc Phe	aat Asn	. 672
	gtg Val 225	cca Pro	ctt Leu	cgc Arg	gta Val	cgc Arg 230	Ser	Ser	tat Tyr	Ser	Asn	Asp	ccc Pro	Gly	act Thr	ttg Leu 240	720
25	att Ile	gcc Ala	ggc	tct Ser	atg Met 245	gag Glu	gat Asp	att Ile	cct Pro	gtg Val 250	gaa Glu	gaa Glu	gca Ala	gtc Val	ctt Leu 255	acc Thr	768
30	ggt Gly	gtc Val	gca Ala	acc Thr 260	gac Asp	aag Lys	tcc Ser	gaa Glu	gcc Ala 265	aaa Lys	gta Val	acc Thr	gtt Val	ctg Leu 270	ggt Gly	att Ile	816
35	tcc Ser	gat Asp	aag Lys 275	cca Pro	ggc Gly	gag Glu	gct Ala	gcg Ala 280	aag Lys	gtt Val	ttc Phe	cgt Arg	gcg Ala 285	ttg Leu	gct Ala	gat Asp	864
40	gca Ala	gaa Glu 290	atc Ile	aac Asn	att Ile	gac Asp	atg Met 295	gtt Val	ctg Leu	cag Gln	aac Asn	gtc Val 300	tct Ser	tct Ser	gta Val	gaa Glu	912
	gac Asp 305	ggc	acc Thr	acc Thr	gac Asp	atc Ile 310	acc Thr	ttc Phe	acc Thr	tgc Cys	cct Pro 315	cgt Arg	tcc Ser	gac Asp	ggc Gly	cgc Arg 320	960
45	cgc Arg	gcg Ala	atg Met	gag Glu	atc Ile 325	ttg Leu	aag Lys	aag Lys	ctt Leu	cag Gln 330	gtt Val	cag Gln	ggc	aac Asn	tgg Trp 335	acc Thr	1008
50	aat Asn	gtg Val	ctt Leu	tac Tyr 340	gac Asp	gac Asp	cag Gln	gtc Val	ggc Gly 345	aaa Lys	gtc Val	tcc Ser	ctc Leu	gtg Val 350	ggt Gly	gct Ala	1056
55	ggc Gly	atg Met	aag Lys 355	tct Ser	cac His	cca Pro	ggt Gly	gtt Val 360	acc Thr	gca Ala	gag Glu	ttc Phe	atg Met 365	gaa Glu	gct Ala	ctg Leu	1104
60	cgc Arg	gat Asp 370	gtc Val	aac Asn	gtg Val	aac Asn	atc Ile 375	gaa Glu	ttg Leu	att Ile	tcc Ser	acc Thr 380	tct Ser	gag Glu	att Ile	cgt Arg	1152

							-	-	_						cgt Arg		1200
5						· · · · · · · · · · · · · · · · · · ·	_								gtt Val 415		1248
10	_			gga Gly 420	_						-						1263
15	<211 <212 <213		121 PRT Coryn	nebad	cteri	ium ç	gluta	amic	um								
	<400)> :	2														
20	Met 1	Ala	Leu	Val	Val 5	Gln	Lys	Tyr	Gly	Gly 10	Ser	Ser	Leu	Glu	Ser 15	Ala	
25	Glu	Arg	Ile	Arg 20	Asn	Val	Ala	Glu	Arg 25	Ile	Va1	Ala	Thr	Lys 30	Lys	Ala	
	Gly	Asn	Asp 35	Val	Val	Val	Val	Cys 40	Ser	Ala	Met	Gly	Asp 45	Thr	Thr	Asp	
30	Glu	Leu 50	Leu	Glu	Leu	Ala	Ala 55	Ala	Val	Asn	Pro	Val 60	Pro	Pro	Ala	Arg	
	Glu 65	Met	Asp	Met	Leu	Leu 70	Thr	Ala	Gly	Glu	Arg 75	Ile	Ser	Asn	Ala	Leu 80	
35	Val	Ala	Met	Ala	11e 85	Glu	Ser	Leu	Gly	Ala 90	Glu	Ala	Gln	Ser	Phe 95	Thr	
40	Gly	Ser	Gln	Ala 100		Val	Leu	Thr	Thr 105		Arg	His	Gly	Asn 110	Ala	Arg	
40	Ile	Val	Asp 115		Thr	Pro	Gly	Arg 120		Arg	Glu	Ala	Leu 125	Asp	Glu	Gly	
45	Lys	Ile 130		Ile	Val	Ala	Gly 135		Gln	Gly	Va1	Asn 140	Lys	Glu	Thr	Arg	
	Asp 145		Thr	Thr	Leu	Gly 150		Gly	Gly	Ser	Asp 155		Thr	Ala	Val	Ala 160	
50	Leu	Ala	. Ala	Ala	Leu 165		Ala	Asp	Val	Cys 170		Ile	Tyr	Ser	Asp 175	Val	
EE	Asp	Gly	Val	. Tyr 180		Ala	Asp	Pro	Arg 185		Va1	Pro	Asn	Ala 190	Gln	Lys	
55	Leu	Glu	Lys 195		Ser	Phe	Glu	G1v 200		Leu	Glu	Leu	A1a 205		Val	Gly	
60	Ser	Lys 210		e Lev	ı Val	Leu	Arg 215		. Val	Glu	Tyr	Ala 220	•	Ala	Phe	Asn	

Val Pro Leu Arg Val Arg Ser Ser Tyr Ser Asn Asp Pro Gly Thr Leu Ile Ala Gly Ser Met Glu Asp Ile Pro Val Glu Glu Ala Val Leu Thr Gly Val Ala Thr Asp Lys Ser Glu Ala Lys Val Thr Val Leu Gly Ile Ser Asp Lys Pro Gly Glu Ala Ala Lys Val Phe Arg Ala Leu Ala Asp Ala Glu Ile Asn Ile Asp Met Val Leu Gln Asn Val Ser Ser Val Glu Asp Gly Thr Thr Asp Ile Thr Phe Thr Cys Pro Arg Ser Asp Gly Arg Arg Ala Met Glu Ile Leu Lys Lys Leu Gln Val Gln Gly Asn Trp Thr Asn Val Leu Tyr Asp Asp Gln Val Gly Lys Val Ser Leu Val Gly Ala Gly Met Lys Ser His Pro Gly Val Thr Ala Glu Phe Met Glu Ala Leu Arg Asp Val Asn Val Asn Ile Glu Leu Ile Ser Thr Ser Glu Ile Arg Ile Ser Val Leu Ile Arg Glu Asp Asp Leu Asp Ala Ala Arg Ala Leu His Glu Gln Phe Gln Leu Gly Gly Glu Asp Glu Ala Val Val Tyr Ala Gly Thr Gly Arg <210> 3 <211> 1263 <212> DNA <213> Corynebacterium glutamicum <220> <221> CDS <222> (1)..(1263) <223> lysC-fbr allele lysC T311I <400> 3 gtg gcc ctg gtc gta cag aaa tat ggc ggt tcc tcg ctt gag agt gcg Met Ala Leu Val Val Gln Lys Tyr Gly Gly Ser Ser Leu Glu Ser Ala gaa cgc att aga aac gtc gct gaa cgg atc gtt gcc acc aag aag gct Glu Arg Ile Arg Asn Val Ala Glu Arg Ile Val Ala Thr Lys Lys Ala gga aat gat gtc gtg gtt gtc tgc tcc gca atg gga gac acc acg gat Gly Asn Asp Val Val Val Cys Ser Ala Met Gly Asp Thr Thr Asp

	gaa Glu	ctt Leu 50	cta Leu	gaa Glu	ctt Leu	Ala	gcg Ala 55	gca Ala	gtg Val	aat Asn	ccc Pro	gtt Val 60	ccg Pro	cca Pro	gct Ala	cgt Arg	Į	192
5	gaa Glu 65	atg Met	gat Asp	atg Met	ctc Leu	ctg Leu 70	act Thr	gct Ala	ggt Gly	gag Glu	cgt Arg 75	att Ile	tct Ser	aac Asn	gct Ala	ctc Leu 80	:	240
10	gtc Val	gcc Ala	atg Met	gct Ala	att Ile 85	gag Glu	tcc Ser	ctt Leu	ggc	gca Ala 90	gaa Glu	gcc Ala	caa Gln	tct Ser	ttc Phe 95	acg Thr		288
15	GJA	tct Ser	cag Gln	gct Ala 100	ggt Gly	gtg Val	ctc Leu	acc Thr	acc Thr 105	gag Glu	ege Arg	cac His	gga Gly	aac Asn 110	gca Ala	cgc Arg		336
20	Ile	Val	Asp 115	Val	Thr	cca Pro	Gly	Arg 120	Val	Arg	Glu	Ala	Leu 125	Asp	Glu	Gly		384
	aag Lys	atc Ile 130	Cys	att Ile	gtt Val	gct Ala	ggt Gly 135	ttc Phe	cag Gln	ggt Gly	gtt Val	aat Asn 140	Lys	gaa Glu	acc Thr	cgc Arg		432
25	gat Asp 145	Val	acc Thr	acg Thr	ttg Leu	ggt Gly 150	cgt Arg	ggt Gly	ggt Gly	tct Ser	gac Asp 155	acc Thr	act	gca Ala	gtt Val	gcg Ala 160		480
30	ttg Leu	gca Ala	gct Ala	gct Ala	ttg Leu 165	aac Asn	gct Ala	gat Asp	gtg Val	tgt Cys 170	Glu	att Ile	tac Tyr	tcg Ser	gac Asp 175	Val		528
35	gac Asp	ggt Gly	gtg Val	tat Tyr 180	Thr	gct Ala	gac Asp	ccg Pro	cgc Arg 185	Ile	gtt Val	cct Pro	aat Asn	gca Ala 190	Gln	aag Lys		576
40	ctg Leu	gaa Glu	aag Lys 195	Leu	agc Ser	ttc Phe	gaa Glu	gaa Glu 200	Met	ctg Leu	gaa Glu	ctt Leu	gct Ala 205	Ala	gtt Val	ggc Gly		624
±0	tcc Ser	aac Lys 210	: Ile	ttg Leu	gtg Val	ctg Leu	cgc Arg 215	Ser	gtt Val	gaa Glu	tac Tyr	gct Ala 220	Arg	gca Ala	ttc Phe	aat Asn		672
45	gtg Val 225	Pro	ctt Lev	cgc Arg	gta Val	cgc Arg 230	Ser	tct Ser	tat Tyr	agt Ser	aat Asn 235	Asp	ccc Pro	ggc	act Thr	ttg Leu 240		720
50	att Ile	gco Ala	e ggo	tct Ser	ato Met	: Glu	gat Asp	att	cct Pro	gtg Val 250	. Glu	gaa Glu	gca Ala	gtc Val	ctt Leu 255	acc Thr		768
55	ggt Gl <u>y</u>	gto Val	e gea l Ala	a acc a Thr 260	c Asp	aag Lys	tco Ser	gaa Glu	a gco a Ala 265	a Lys	gta Val	acc Thr	gtt Val	cto Lev 270	ı Gly	att Ile		816
60	tco Sei	c gat	t aaq 5 Lys 27!	s Pro	a ggo o Gly	gag Y Glu	g gct 1 Ala	gcg A Ala 280	a Lys	g gtt s Val	tto Phe	c cgt	gcg Ala 285	Lev	gct Ala	gat Asp		864
60																		

	-	_		aac Asn		_	_	_	_	_		_				_	912
5				acc Thr	_									_			960
10	_		_	gag Glu		-	_										1008
15				tac Tyr 340													1056
20				tct Ser												ctg Leu	1104
				aac Asn													1152
25				ctg Leu													1200
30				cag Gln													1248
35				gga Gly 420	Arg												1263
4.0		1> 2>	4 421 PRT	3		•											
40	<21 <40	<i>3></i> 0>	_	neba	ccer.	raiii	grac	amrc	wii.								
45	Met 1	Ala	Leu	Val	Val 5	Gln	Lys	Tyr	Gly	Gly 10	Ser	Ser	Leu	Glu	Ser 15	Ala	
	Glu	Arg	Ile	Arg 20	Asn	Val	Ala	Glu	Arg 25	Ile	Val	Ala	Thr	Lys 30	Lys	Ala	
50	Gly	Asn	Asp 35	Val	Va1	Val	Val	Суs 40	Ser	Ala	Met	Gly	Asp 45	Thr	Thr	Asp	
55	Glu	Leu 50	Leu	Glu	Leu	Ala	Ala 55	Ala	. Val	Asn	Pro	Val 60	Pro	Pro	Ala	Arg	
<i>J J</i>	G1u 65	Met	: Asp	Met	Leu	Leu 70	Thr	· Ala	Gly	Glu	Arg 75	Ile	Ser	Asn	Ala	Leu 80	
60	Val	Ala	Met	. Ala	Ile 85	Glu	Ser	Leu	Gly	Ala 90	Glu	Ala	Gln	Ser	Phe 95	Thr	

	Gly	Ser	Gln	Ala 100	Gly	Val	Leu	Thr	Thr 105	Glu	Arg	His	Gly	Asn 110	Ala	Arg
5	Ile	Val	Asp 115	Val	Thr	Pro	Gly	Arg 120	Val	Arg	Glu	Ala	Leu 125	Asp	Glu	Gly
	Lys	Ile 130	Cys	Ile	Va1	Ala	Gly 135	Phe	Gln	Gly	Val	Asn 140	Lys	Glu	Thr	Arg
10	Asp 145	Val	Thr	Thr	Leu	Gly 150	Arg	Gly	Gly	Ser	Asp 155	Thr	Thr	Ala	Val	Ala 160
15	Leu	Ala	Ala	Ala	Leu 165	Asn	Ala	Asp	Val	Cys 170	Glu	Ile	Tyr	Ser	Asp 175	Val
	Asp	Gly	Val	Tyr 180	Thr	Ala	Asp	Pro	Arg 185	Ile	Val	Pro	Asn	Ala 190	Gln	Lys
20	Leu	Glu	Lys 195	Leu	Ser	Phe	Glu	Glu 200	Met	Leu	Glu	Leu	A1a 205	Ala	Val	Gly
	Ser	Lys 210	Ile	Leu	Val	Leu	Arg 215	Ser	Val	Glu	Tyr	Ala 220	Arg	Ala	Phe	Asn
25	Val 225	Pro	Leu	Arg	Val	Arg 230		Ser	Tyr	Ser	Asn 235	Asp	Pro	Gly	Thr	Leu 240
30	Ile	Ala	Gly	Ser	Met 245	Glu	Asp	Ile	Pro	Val 250	Glu	Glu	Ala	Val	Leu 255	Thr
	Gly	Val	Ala	Thr 260	Asp	Lys	Ser	Glu	Ala 265	Lys	Val	Thr	Val	Leu 270	Gly	Ile
35	Ser	Asp	Lys 275	Pro	Gly	Glu	Ala	Ala 280	Lys	Val	Phe	Arg	Ala 285	Leu	Ala	Asp
4.0	Ala	Glu 290	Ile	Asn	Ile	Asp	Met 295	Val	Leu	Gln	Asn	Val 300	Ser	Ser	Val	Glu
40	Asp 305	Gly	Thr	Thr	Asp	Ile 310	Ile	Phe	Thr	Cys	Pro 315	Arg	Ser	Asp	Gly	Arg 320
45	Arg	Ala	Met	Glu	Ile 325	Leu	Lys	Lys	Leu	Gln 330	Val	Gln	Gly	Asn	Trp 335	Thr
	Asn	Val	Leu	Tyr 340	Asp	qaA	Gln	Val	Gly 345	Lys	Val	Ser	Leu	Val 350	Gly	Ala
50	Gly	Met	Lys 355	Ser	His	Pro	Gly	Val 360	Thr	Ala	Glu	Phe	Met 365	Glu	Ala	Leu
	Arg	Asp 370	Val	Asn	Val	Asn	Ile 375	Glu	Leu	Ile	Ser	Thr 380	Ser	Glu	Ile	Arg
55	Ile 385	Ser	Va1	Leu	Ile	Arg 390	Glu	Asp	Asp	Leu	Asp 395	Ala	Ala	Ala	Arg	Ala 400
60	Leu	His	Glu	Gln	Phe 405	Gln	Leu	Gly	Gly	Glu 410	Asp	Glu	Ala	Val	Val 415	Tyr
	Ala	Gly	Thr	Gly 420	Arg											

```
<210> 5
    <211> 28
    <212> DNA
5 <213> Artificial sequence
     <220>
     <221> misc_feature
    <222> (1)..(28)
10 <223> Primer lysC1beg
     <400> 5
     taggatecte eggtgtetga ceaeggtg
                                                                      28
15
    <210> 6
    <211> 29
     <212> DNA
     <213> Artificial sequence
20
     <220>
     <221> misc_feature
     <222> (1)..(29)
     <223> Primer lysC2end
25
    <400> 6
                                                                      29
     acggatccgc tgggaaattg cgctcttcc
     <210> 7
     <211> 28
30
     <212> DNA
     <213> Artificial sequence
     <220>
     <221> misc_feature
35
     <222> (1)..(28)
     <223> Primer gluBg11
     <400> 7
                                                                       28
     taagatctgt gttggacgtc atggcaag
40
     <210> 8
     <211> 28
     <212> DNA
     <213> Artificial sequence
45
     <220>
     <221> misc_feature
     <222> (1)..(28)
     <223> Primer gluBgl2
50
     <400> 8
                                                                       28
     acagatettg aagecaagta eggecaag
     <210> 9
55
     <211> 27
     <212> DNA
     <213> Artificial sequence
     <220>
     <221> misc_feature
60
     <222> (1)..(27)
      <223> Primer pck_beg
```

	<400> 9 taagatetge eggeatgaet teagttt	27
5	<210> 10 <211> 30 <212> DNA <213> Artificial sequence	
10	<pre><220> <221> misc_feature <222> (1)(30) <223> Primer pck_end</pre>	
15	<400> 10 acagatctgg tgggagcctt tcttgttatt	30
20	<210> 11 <211> 20 <212> DNA <213> Corynebacterium glutamicum	
25	<pre><220> <221> misc_feature <222> (1)(20) <223> Primer aecD_beg</pre>	
30 35	<400> 11 gaacttacgc caagctgttc <210> 12 <211> 20 <212> DNA <213> Corynebacterium glutamicum	20
40	<220> <221> misc_feature <222> (1)(20) <223> Primer aecD_end <400> 12 agcaccacaa tcaacgtgag	20
45	<210> 13 <211> 20 <212> DNA <213> Corynebacterium glutamicum	
50	<220> <221> misc_feature <222> (1)(20) <223> Primer gluA_beg	
55	<400> 13 cacggttgct cattgtatcc	20
60	<210> 14 <211> 20 <212> DNA <213> Corynebacterium glutamicum	

<220> <221> misc_feature <222> (1)..(20) <223> Primer gluD_end <400> 14 cgaggcgaat cagacttctt 20 <210> 15 10 <211> 20 <212> DNA <213> Corynebacterium glutamicum <220> 15 <221> misc_feature <222> (1)..(20) <223> Primer ddh_beg <400> 15 20 ctgaatcaaa ggcggacatg 20 <210> 16 <211> 20 <212> DNA 25 <213> Corynebacterium glutamicum <220> <221> misc_feature <222> (1)..(20) 30 <223> Primer ddh_end <400> 16 tcgagctaaa ttagacgtcg 20 35 <210> 17 <211> 20 <212> DNA <213> Corynebacterium glutamicum 40 <220> <221> misc_feature <222> (1)..(20) <223> Primer dapA_beg 45 <400> 17 cgagccagtg aacatgcaga 20 <210> 18 <211> 20 50 <212> DNA <213> Corynebacterium glutamicum <220> <221> misc_feature 55 <222> (1)..(20) <223> Primer dapA_end <400> 18 cttgagcacc ttgcgcagca

60

. 20

PCT/EP02/08464 WO 03/040373

```
<210> 19
     <211> 28
     <212> DNA
     <213> Artificial sequence
     <220>
     <221> misc_feature
     <222> (1)..(28)
     <223> Primer pyc_beg
10
     <400> 19
                                                                         28
     tcacgcgtct tgaagtcgtg caggtcag
     <210> 20
15
     <211> 28
     <212> DNA
     <213> Artificial sequence
     <220>
20 <221> misc_feature
     <222> (1)..(28)
     <223> Primer pyc_end
     <400> 20
25 tcacgcgtcg cctcctccat gaggaaga
                                                                         28
     <210> 21
     <211> 39
     <212> DNA
30
     <213> Corynebacterium glutamicum
     <220>
     <221> misc_feature
     <222> (1)..(39)
35
     <223> Primer P458S-1
     <400> 21
     ggattcattg ccgatcactc gcacctcctt caggctcca
40
     <210> 22
     <211> 39
     <212> DNA
     <213> Corynebacterium glutamicum
45
     <220>
     <221> misc_feature
     <222> (1)..(39)
     <223> Primer P458S-2
50
     <400> 22
                                                                         39
     gtggaggaag tccgaggtcg agtgatcggc aatgaatcc
```

(19) World Intellectual Property Organization International Bureau





(43) International Publication Date 15 May 2003 (15.05.2003)

PCT

(10) International Publication Number WO 03/040373 A3

- (51) International Patent Classification⁷: C12N 15/52, 15/53, 15/54, 15/60, C12P 13/08, C12N 1/21 // (C12P 13/08, C12R 1:15)
- (21) International Application Number: PCT/EP02/08464
- (22) International Filing Date: 30 July 2002 (30.07.2002)
- (25) Filing Language:

English

(26) Publication Language:

English

US

- (30) Priority Data:
- (71) Applicant (for all designated States except US): DE-GUSSA AG [DE/DE]; Bennigsenplatz 1, 40474 Düsseldorf (DE).

6 August 2001 (06.08.2001)

(72) Inventors; and

60/309,878

(75) Inventors/Applicants (for US only): BATHE,

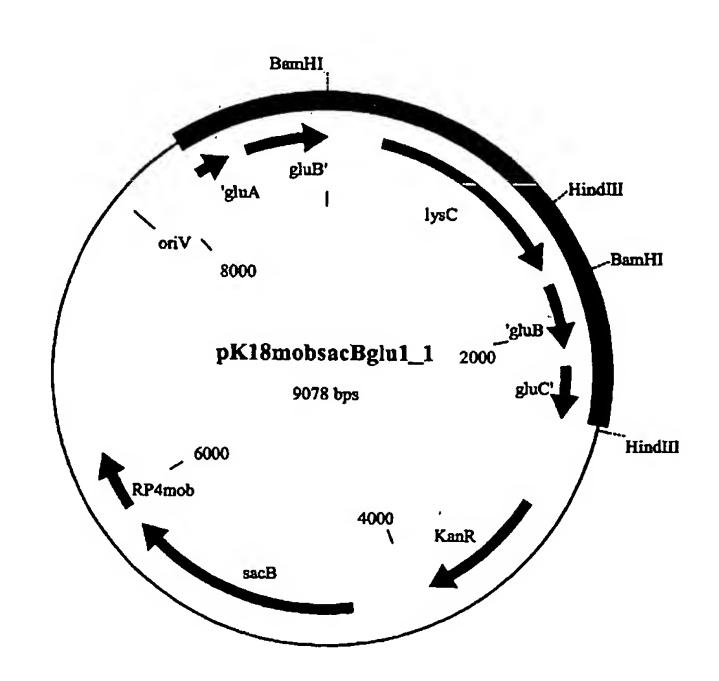
Brigitte [DE/DE]; Twieten 1, 33154 Salzkotten (DE). KREUTZER, Caroline [DE/DE]; Poststrasse 16, 49326 Melle (DE). MÖCKEL, Bettina [DE/DE]; Benrodestrasse 35, 40597 Düsseldorf (DE). THIERBACH, Georg [DE/DE]; Gunststrasse 21, 33613 Bielefeld (DE).

- (74) Common Representative: DEGUSSA AG; Intellectual Property Management, Patents And Trademarks, Location Hanau, P.O. Box 13 45, 63403 Hanau (DE).
- (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),

[Continued on next page]

(54) Title: PRODUCTION OF L-LYSINE BY GENETICALLY MODIFIED CORYNEBACTERIUM GLUTAMICUM STRAINS

Plasmid pK18mobsacBglu1_1



(57) Abstract: The invention relates to coryneform bacteria which have, in addition to at least one copy, present at the natural site (locus), of an open reading frame (ORF), gene or allele which codes for the synthesis of a protein or an RNA, in each case a second, optionally third or fourth copy of this open reading frame (ORF), gene or allele at in each case a second, optionally third or fourth site in a form integrated into the chromosome and processes for the preparation of chemical compounds by fermentation of these bacteria.

WO 03/040373 A.



European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW, ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent

(AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)

— of inventorship (Rule 4.17(iv)) for US only

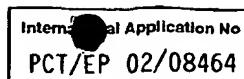
Published:

— with international search report

(88) Date of publication of the international search report:

18 December 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

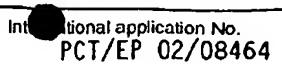


A. CLASSIFICATION OF SUBJECT MATTER IPC 7 C12N15/52 C12N C12P13/08 C12N15/60 IPC 7 C12N15/53 C12N15/54 //(C12P13/08,C12R1:15) C12N1/21 According to International Patent Classification (IPC) or to both national classification and IPC B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 7 C12N C12P Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, WPI Data, PAJ, SEQUENCE SEARCH, BIOSIS, EMBASE C. DOCUMENTS CONSIDERED TO BE RELEVANT Relevant to claim No. Category ° Citation of document, with indication, where appropriate, of the relevant passages 1 - 38,40KRONEMEYER, W. ET AL.: "Structure of the gluABCD Cluster Encoding the Glutamate Uptake System of Corynebacterium glutamicum" JOURNAL OF BACTERIOLOGY, vol. 177, no. 5, March 1995 (1995-03), pages 1152-1158, XP002935147 cited in the application abstract page 1154; figure 2 page 1157, column 1, line 9 - line 13 Further documents are listed in the continuation of box C. Patent family members are listed in annex. Special categories of cited documents: *T* later document published after the international filing date or priority date and not in conflict with the application but "A" document defining the general state of the art which is not cited to understand the principle or theory underlying the considered to be of particular relevance invention "E" earlier document but published on or after the International "X" document of particular relevance; the claimed invention filing date cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another "Y" document of particular relevance; the claimed invention cliation or other special reason (as specified) cannot be considered to involve an inventive step when the *O' document referring to an oral disclosure, use, exhibition or document is combined with one or more other such documents, such combination being obvious to a person skilled other means in the art. *P* document published prior to the international filing date but *&* document member of the same patent family later than the priority date claimed Date of mailing of the international search report Date of the actual completion of the international search 08 08 2003 25 July 2003 Authorized officer Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fuchs, U

Fax: (+31-70) 340-3016

PCT/EP 02/08464

	PC1/EP 02/08464
y ^a	Relevant to claim No.
ISHINO, S. ET AL.: "Nucleotide sequence of the meso-diaminopimelate D-dehydrogenase gene from Corynebacterium glutamicum" NUCLEIC ACIDS RESEARCH, vol. 15, no. 9, 11 May 1987 (1987-05-11), page 3917 XP001056569 cited in the application the whole document	1-37
EP 1 094 111 A (DEGUSSA-HÜLS AG) 25 April 2001 (2001-04-25) cited in the application the whole document see especially: page 9 -page 10; examples 5,6; tables 2,3	1-37
EP 1 067 192 A (DEGUSSA-HÜLS AG; FORSCHUNGSZENTRUM JÜLICH GMBH) 10 January 2001 (2001-01-10) the whole document see especially: page 12 -page 13; examples 12-15 and page 19; claims 1,7-9,11-15,19,20	1-40
EP 1 108 790 A (KYOWA HAKKO KOGYO CO., LTD.) 20 June 2001 (2001-06-20) cited in the application the whole document see especially: page 224 -page 228; examples 2,3	1-40
WO 00 63388 A (KYOWA HAKKO KOGYO CO., LTD.) 26 October 2000 (2000-10-26) cited in the application abstract SEQ ID NOS: 17 and 18	1-40
-& EP 1 172 437 A (KYOWA HAKKO KOGYO CO., LTD) 16 January 2002 (2002-01-16) the whole document	1-40
SCHÄFER, A. ET AL.: "Small mobilizable multi-purpose cloning vectors derived from the Escherichia coli plasmids pK18 and pK19: selection of defined deletions in the chromosome of Corynebacterium glutamicum" GENE, vol. 145, no. 1, 22 July 1994 (1994-07-22), pages 69-73, XP001093898 cited in the application the whole document	1-40
	ISHINO, S. ET AL.: "Nucleotide sequence of the meso-diaminopimelate D-dehydrogenase gene from Corynebacterium glutamicum" NUCLEIC ACIDS RESEARCH, vol. 15, no. 9, 11 May 1987 (1987-05-11), page 3917 XP001056569 cited in the application the whole document EP 1 094 111 A (DEGUSSA-HÜLS AG) 25 April 2001 (2001-04-25) cited in the application the whole document see especially: page 9 -page 10; examples 5,6; tables 2,3 EP 1 067 192 A (DEGUSSA-HÜLS AG; FORSCHUNGSZENTRUM JÜLICH GMBH) 10 January 2001 (2001-01-10) the whole document see especially: page 12 -page 13; examples 12-15 and page 19; claims 1,7-9,11-15,19,20 EP 1 108 790 A (KYOWA HAKKO KOGYO CO., LTD.) 20 June 2001 (2001-06-20) cited in the application the whole document see especially: page 224 -page 228; examples 2,3 WO 00 63388 A (KYOWA HAKKO KOGYO CO., LTD.) 26 October 2000 (2000-10-26) cited in the application abstract SEQ ID NOS: 17 and 18 page 24 -page 28 -& EP 1 172 437 A (KYOWA HAKKO KOGYO CO., LTD.) 16 January 2002 (2002-01-16) the whole document SCHÄFER, A. ET AL.: "Small mobilizable multi-purpose cloning vectors derived from the Escherichia coli plasmids pK18 and pK19: selection of defined deletions in the chromosome of Corynebacterium glutamicum" GENE, vol. 145, no. 1, 22 July 1994 (1994-07-22), pages 69-73, XP001093898 cited in the application



Box I	Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)
This Inte	ernational Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:
1.	Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
2. X	Claims Nos.: 1-37 because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically: see FURTHER INFORMATION sheet PCT/ISA/210
з	Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).
Box II	Observations where unity of invention is lacking (Continuation of item 2 of first sheet)
This Inte	emational Searching Authority found multiple inventions in this international application, as follows:
	see additional sheet
1. X 2 3	As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims. As all searchable daims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.: No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the Invention first mentioned in the claims; it is covered by claims Nos.:
Remark	The additional search fees were accompanied by the applicant's protest. No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-37 partially and 38, 40 completely

Corynebacterium glutamicum strains DSM13994glu::lysC (see example 1.3), DSM12866glu::lysC (see example 1.4) and DSM12866glu::ddh (see example 2.2), a process for the preparation of lysine involving said strains, a process for the production of said strains, plasmid pK18mobsacBglul_1;

2. Claims: 1-37 partially

Corynebacterium glutamicum strains DSM12866pck::lysC (see example 1.6) and DSM12866pck::pyc (see example 4.3), a process for the preparation of lysine involving said strains, a process for the production of said strains;

3. Claims: 1-37 partially and 39 completely

Corynebacterium glutamicum strain DSM12866aecD::lysC (see example 1.8), a process for the preparation of lysine involving said strain, a process for the production of said strain, plasmid pK18mobsacSaecD1_1;

4. Claims: 1-37 partially

Corynebacterium glutamicum strain DSM12866aecD::dapA (see example 3.2), a process for the preparation of lysine involving said strain, a process for the production of said strain.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-37

Present claims 1-19 relate to coryneform bacteria defined by reference to desirable characteristics or properties, namely "which produce chemical compounds", "wherein these have, in addition to at least one copy, present at the natural site (locus), of an open reading frame (ORF), gene or allele which codes for the synthesis of a protein or an RNA, a second, optionally third or fourth copy of the open reading frame (ORF), gene or allele in question", "at a second, optionally third or fourth site in a form integrated into the chromosome ... and the second, optionally third or fourth site not relating to open reading frames (ORF), genes or alleles which are essential for the growth of the bacteria and the production of the desired compound". Furthermore, claim 37 relates to a process for the production of such bacteria.

The claims cover all coryneform bacteria having these characteristic or properties, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such bacteria. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the bacteria by reference to results to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the Corynebacterium glutamicum strains produced in examples 1.2-4.3.

The same applies to claims 20-36 relating to a process for the preparation of chemical compounds involving such bacteria. Again, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the preparation of lysine as described in example 5.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

incormation on patent family members

PCT/EP 02/08464

Patent document cited in search report		Publication date		Patent family member(s)	Publication date
EP 1094111		25-04-2001	DE	19950409 A1	26-04-2001
200122	••	20 0 . 200	AU	6410400 A	26-04-2001
			BR	0004957 A	29-05-2001
			CA	2322555 A1	20-04-2001
			CN	1308125 A	15-08-2001
			EP	1094111 A2	25-04-2001
			HU	0004115 A2	28-09-2002
			JP	2001149086 A	05-06-2001
			PL	343339 A1	23-04-2001
			SK	15292000 A3	03-12-2001
			US	2003003548 A1	02-01-2003
			US	2002065403 A1	30-05-2002
EP 1067192	A	10-01-2001	DE	19931317 A1	11-01-2001
			AU	4509900 A	11-01-2001
			BR	0002655 A	05-06-2001
			CA	2310870 A1	07-01-2001
			CN	1280184 A	17-01-2001
			ΕP	1067192 A1	10-01-2001
			HU	0002585 A2	28-09-2002
			JP	2001061485 A	13-03-2001
			SK	10142000 A3	09-04-2001
			US	6200785 B1	13-03-2001
			US	2002086371 A1	04-07-2002
EP 1108790	A	20-06-2001	EP	1108790 A2	20-06-2001
			JP	2002191370 A	09-07-2002
			US	2002197605 A1	26-12-2002
WO 0063388	Α	26-10-2000	AU	3837400 A	02-11-2000
			CN	1355849 T	26-06-2002
			EP	1172437 A1	16-01-2002
			WO	0063388 A1	26-10-2000